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N-Cadherin in Prostate Cancer: Downstream Pathways and their Translational Application for Castrate-Resistant Prostate Cancer

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14. ABSTRACT
Castration resistant prostate cancer (CRPC) is responsible for the overwhelming majority of prostate-cancer specific deaths. Although continued activation of the androgen receptor (AR) plays an important role in the development of castration resistance, AR-independent mechanisms represent an alternative source of biochemical signals that drive castration resistance. We have identified heightened expression of N-cadherin as a driving force in invasion, metastasis and castration resistance. Here we sought to identify biochemical signals that are regulated by N-cadherin in CRPC cells that can mediate the biologic effects of N-cadherin. In addition to activation of NF-kappa B reported in our first annual report, we have now identified the c-Jun N-terminal kinase (JNK) MAP kinase pathway as a signaling pathway that is activated in response to N-cadherin expression. We have generated inducible N-cadherin shRNA models to study the biochemical and cell biologic effects of N-cadherin. We have shown that N-cadherin activates JNK, which in turn phosphorylates the activator protein 1 (AP1) transcription factor c-Jun. Phosphorylated c-Jun then heterodimerizes with c-Fos and drives expression of proteins including Twist, that drive an epithelial-to-mesenchymal transition with heightened invasiveness. Heterodimers of c-Jun and c-Fos directly bind to the *Twist* promoter to drive *Twist* gene transcription. Pharmacologic inhibition of JNK reversed the effects of N-cadherin on invasion. Thus, in addition to NF-kappa B, the JNK pathway serves as another potential target for intervention. The MAPKK that activates JNK is MKK4, which in turn is activated by the MAPKKK, TAK1. Importantly, TAK1 is the MAPKKK that activates the IKK complex in the NF-kappa B pathway; inhibition of TAK1 therefore blocks both NF-kappa B and JNK activation that occurs downstream of N-cadherin and serves as a potential therapeutic target.

15. SUBJECT TERMS
Prostate cancer; N-cadherin; c-Jun N-terminal kinase; castration resistance.

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Table of Contents

INTRODUCTION:

Metastatic castrate-resistant prostate cancer (CRPC) accounts for the vast majority of prostate cancer related deaths. Experimental and clinical evidence support the notion that reactivation of the androgen receptor (AR) seems to play a critical role in the emergence of the castrate-resistant state, and intensive investigative efforts have focused on the development of drugs that inhibit the androgen-AR signaling axis in unique and more effective ways (1). Abiraterone acetate, an inhibitor of the CYP17 enzyme complex that mediates that rate limiting steps in androgen synthesis, irrespective of their source, including the tumor cell itself, received FDA approval for patients with metastatic, castration resistant prostate cancer who have already received docetaxel-based chemotherapy (2). MDV3100, a potent androgen receptor antagonist, was recently reported to improve survival in post-chemotherapy metastatic CRPC. However, continued activation of AR signaling does not account for all cases of CRPC. Identification and targeting of the signaling pathways that promote AR-independent growth will be critical to advancing the treatment of CRPC (3, 4).

The theme of this proposal is centered around the role of increased N-cadherin expression in CRPC. We have previously credentialed N-cadherin as a viable target in CRPC, in that it is over-expressed in CRPC and its expression stimulates invasion, epithelial to mesenchymal transition (EMT), and tumorigenesis and promotes castration resistant growth (5). To build on these findings, we have sought to identify pathways that may mediate and or function in conjunction with N-cadherin to promote prostate cancer growth. In our first year progress report, we emphasized the role of activation of the NF-κB pathway as a downstream mediator of N-cadherin. In addition, we identified AKT as an intermediate that delivers signals from N-cadherin to the NF-κB pathway.

JNK is a mitogen activated protein kinase (MAPK) that phosphorylates the Jun family of transcription factors, which homo- and hetero-dimerize to form transcriptional complexes known as activator protein 1 (AP1) transcription factors (6). The literature has established an important role for the JNK/AP1 signaling pathway in oncogenesis (7, 8). For example, Jun is required Ras-induced transformation, and c-Jun is required for chemically induced hepatocellular carcinoma. In addition, the JNK and NF-κB share common upstream biochemical signals. Here, we describe the N-cadherin-dependent activation of the c-Jun N-terminus kinase (JNK) signaling pathway and provide evidence for its pathophysiologic role in prostate cancer growth.

BODY:

Task 1: Identify the biochemical signaling effectors of N-cadherin (Months 1-18; Rettig and Reiter).

a. Establish the specific IKK isoforms and NF-κB family members activated by N-cadherin (Months 1-3; Rettig)

   We have shown that prostate cancer cell lines that express N-cadherin have high NF-κB activation as compared to the N-cadherin non-expressing cell lines (e.g. LNCaP). Specifically, we employed isogenic lines of LNCaP cells, the parental cell line of which was transduced with an empty retroviral vector whereas the experimental group was transduced with a retroviral vector for stable expression of wild-type N-cadherin. Clones were selected for expression of N-cadherin that

approximated that of the expression of prostate cancer cells lines that exhibit endogenous expression of N-cadherin. Ectopic expression of N-cadherin in LNCaP cells is sufficient to activate NF-κB (Fig 1A; appendix).

The baseline level of NF-κB was assessed with several assays. Using electrophoretic mobility assays, we observed constitutive binding of NF-κB activation as manifested by heightened binding of NF-κB families members to a consensus NF-κB DNA binding site (Fig 1B; appendix). Moreover, *in vitro* kinase assays documented that the isoform of IKK that is operative in cell lines that express N-cadherin is the IKKβ isoform (Fig. 1C; appendix). Electrophoretic mobility shift assays demonstrated that the NF-κB family members that are operative in the NF-κB complex are p65 and p50 (not shown), dimers that are representative of the classical pathway for which IKKβ is required for activation.

Because we have shown that N-cadherin is associated with heightened invasion, we sought to determine whether the increased NF-κB activation in cells that express N-cadherin. Cells that ectopically express N-cadherin and thereby have higher NF-κB activity manifest increased invasion compared to N-cadherin non-expressors. Inhibition of NF-κB with a selective IKKβ inhibitor reversed the heightened invasiveness of N-cadherin expressing cells (Figures 2 and 3; appendix). In addition, we used a molecular approach to inhibit NF-κB by transducing an adenovirus expressing a dominant active IκB-SR (Inhibitor of kappa B super repressor). As shown in Figures 2 and 3 (appendix), transduction of the Ad-IκB-SR inhibited the invasiveness of N-cadherin expressing cells. The reduced invasiveness resulting from NF-κB blockade was associated with increased expression of E-cadherin, a marker for an epithelial phenotype typified by reduced invasive potential (Fig. 3D; appendix). In summary, these data indicate that N-cadherin induces NF-κB activity and that inhibition of this increased NF-κB activation significantly reduces the invasiveness of N-cadherin expressing cells. These findings implicate NF-κB activation as an etiologic factor in mediating invasiveness in the setting of N-cadherin expression.

b. Assess the interactions between N-cadherin and NF-κB, AKT and AR signaling . (Months 4-12; Rettig).

In this sub-task, we first evaluated the effects N-cadherin expression on PI3K/AKT and AR signaling activity. As shown in Fig. 4A (appendix), ectopic expression of N-cadherin was associated with increased AKT activity as assayed by an *in vitro* kinase assay. Inhibition of the PI3K/AKT pathway with either pharmacologic PI3K or direct AKT inhibitors resulted in specific inhibition of NF-κB activity as measured by reporter gene assays (Fig. 4B; appendix).

We next evaluated the effects of N-cadherin expression on AR expression. Interestingly, parental cell lines with N-cadherin expression (PC3, CL1) lack AR expression, whereas those cells that lack N-cadherin do express the AR (Fig. 5; appendix). Importantly, ectopic expression of N-cadherin in LNCaP cells abrogated AR expression, a finding that strongly implicates N-cadherin expression as an upstream regulator of AR expression. This *in vitro* finding has been corroborated by *in vivo* results in LAPC9 xenografts passaged in castrated

mice (9). In these *in vivo* experiments, N-cadherin expression was inversely correlated with AR expression as demonstrated by immunofluorescence. Thus, N-cadherin expression results in down-regulation of AR expression both *in vitro* and *in vivo*. However, based on our *in vivo* data, there are likely subpopulations of cells that express both, whereby dual targeting of the AR and N-cadherin may serve as viable therapeutic approaches. This will be investigated in Task 3 and will require the development of models that co-express both AR and N-cadherin.

c. Determine the in vitro biologic effects of inhibiting N-cadherin along with downstream biochemical effectors (Months 13-18; Rettig and Reiter).

We have previously shown that N-cadherin expression enhances invasion and tumorigenesis. To further investigate N-cadherin biochemistry and cell biology, we developed inducible N-cadherin shRNA cell models. Here a lentiviral backbone that expresses N-cadherin specific shRNA under the regulation of a tetracycline-inducible promoter was stably introduced into LNCaP-C2 and PC3 cells. LNCaP-C2 cells stably express retrovirally introduced N-cadherin, whereas PC3 cells endogenously express N-cadherin. We employed two different N-cadherin targeting sequences, which yielded similar results. We show the results from one of the targeting sequences for each cell line. Of note, in generating the stable inducible cell lines, we isolated several clones and examined each clone for inducible knock-down of N-cadherin expression (Figure 6A), and selected one clone for detailed investigation (clone 4 for LNCaP-C2, and clone 3 for PC3). Silencing of N-cadherin expression by the addition of doxycycline markedly reduced the invasion of both LNCaP-C2 and PC3 cells in a Matrigel invasion assay (Figure 6B), a finding that indicts N-cadherin in the invasiveness of prostate cancer. Studies into the role of JNK signaling in invasiveness of prostate cancer cells is presented below.

Task 2: Establish the role of the extracellular and intracellular domains of N-cadherin on EMT, castrate-resistance, and downstream biochemical signaling. (Months 1-18; Reiter and Rettig).

a. Construct N-cadherin/E-cadherin chimeras and generate stable lines. (Months 1-6; Reiter).

   Presented by Dr. Reiter.

b. Assess the contribution of N-cadherin domains on *in vitro* and *in vivo* biologic endpoints, including invasion, metastasis, and androgen independence (Months 7-18; Reiter).

c.

   Presented by Dr. Reiter.

d. Assess the contribution of N-cadherin domains on gene expression and downstream signal transduction (Months 7-18; Reiter and Rettig).

   Presented by initiating PI, Dr. Reiter.

Task 3: Test efficacy of N-cad monoclonal antibodies in combination with inhibitors of biochemical signaling pathways downstream of N-cadherin in murine (Months 19-36; Reiter and Rettig).

a. Test the ability of N-cadherin antibodies alone and in combination with signaling inhibitors to inhibit tumorigenesis, induce regression and block metastasis in castrate mice (Months 19-36; Reiter).

Given the background described in the introduction, we postulated that JNK activity is dysregulated in prostate cancer cells that overexpress N-cadherin. Indeed, LNCaP-C2 cells, which ectopically express N-cadherin, manifest enhanced expression of phosphorylated (Ser 73) c-Jun (Figure 7A, left panels). Moreover, JNK activity, as directly measured by JNK *in vitro* kinase assays, was similarly hyperactivated in LNCaP-C2 cells (Figure 7A, right panels). To determine whether N-cadherin expression was biochemically linked to JNK activation, we silenced N-cadherin expression by exposing LNCaP-C2 and PC3 cells to doxycycline (1 μg/ml). Downregulation of N-cadherin led to reduced phospho-c-Jun and JNK activity (Figures 7B-C). Moreover, increasing concentrations of doxycycline led to a dose-dependent reduction in AP1 reporter activity (Figure 7D), a finding that further supports the link between N-cadherin and downstream JNK/AP1 activation.

b. Identify signaling pathways and biologic processes (e.g. angiogenesis, proliferation apoptosis) affected by above therapies (Months 19-36; Rettig and Reiter).

We next sought to determine mechanisms underlying the N-cadherin-dependent invasiveness and epithelial to mesenchymal transition. Using our tet-inducible cell models, we examined the expression of transcription factors, including Twist, Slug, Zeb1, and Zeb2, which regulate these biologic processes. Silencing N-cadherin expression with the addition of doxycycline, led to a reduction in Twist and Slug expression but no change in Zeb1 or Zeb2 levels (Figure 8A). Importantly, pharmacologic inhibition of JNK activity with SP600125 (hereafter termed JNKi) led to a dose-dependent reduction in Twist expression (Figure 8B). Inhibition of Twist expression by the JNKi was associated reduced invasiveness. In aggregate, these findings implicate AP1-dependent Twist expression in the N-cadherin regulated EMT and invasiveness of prostate cancer cells.

We next investigated the transcriptional regulation of Twist expression by AP1. To generate a map of putative AP1 binding sites in the *Twist* promoter, we employed M-Match, a publically available internet search tool for identifying transcription factor binding sites in DNA sequences. Analysis of the 2.5 kb region upstream of the *Twist* transcription start site identified numerous potential AP1 binding sites with varying homology to the consensus AP1 sequence (Figure 9A). Chromatin immunoprecipitation (ChIP) experiments identified a 350 bp region (-2,589 to –2,238 relative to the transcription start site; hereafter termed the “set 1” segment) containing four predicted AP1 binding sites to which c-Jun was recruited (Figure 9B). The specificity of the PCR signal was validated by the use of several controls: immunoprecipitation with non-specific IgG antibody, PCR amplification with water (no DNA), and PCR amplification with multiple sets of primers from other regions in the *Twist* 5’ UTR (Figure 9B). Because AP1 complexes are often composed of c-Jun and Fos family heterodimers, we predicted that c-Jun forms heterodimers with c-Fos on the AP1 DNA binding sites of the *Twist*

promoter. Indeed, ChIP experiments confirmed that c-Fos was recruited to the set 1 region of the *Twist* promoter in a similar manner as c-Jun (Figure 9B).

We investigated the signaling pathway that mediates N-cadherin activation of JNK. There are two MAPKKs, MKK4 and MKK7, which are known to activate JNK. By Western blot, the phosphorylated MKK7 levels did not differ in PC3 and LNCaP-C2 inducible cell lines with and without doxycycline (Figure 10A). However, suppression of N-cadherin expression by doxycycline led to a reduction in phosphorylated MKK4 levels (Figure 10A). When we transfected a MKK4 dominant negative construct that lack kinase activity, AP1 reporter activity was inhibited in a dose-dependent fashion, a finding that indicates that MKK4 represents the MAPKK that activates JNK in these N-cadherin expressing cancer cell lines (Figure 10B).

To determine the MAPKKK (MAP3K) that activates MKK4/JNK, we hypothesized that N-cadherin expressing prostate cancer cells would employ a common upstream signaling component to activate both the JNK and NF-κB pathways. The MAPKKK known as TAK1 activates the IKKβ complex in the classical NF-κB pathway, which we previously demonstrated to be activated by N-cadherin. Accordingly, we determined whether TAK1 regulates JNK/AP1 activity. As shown in Figure 11A, inhibition of N-cadherin expression by exposure to doxycycline in the inducible cell lines led to decreased phospho-TAK1 expression. Pharmacologic inhibition of TAK1 led to a dose-dependent reduction in AP1 driven reporter activity (Figure 11B). In addition and as expected, TAK1 also inhibited NF-κB driven reporter activity (not shown). Given our findings that both JNK/AP1 and NF-κB regulate EMT of N-cadherin expressing prostate cancer cells, TAK1 represents a favorable target for therapeutic invention.

KEY RESEARCH ACCOMPLISHMENTS:

- NF-κB activation is heightened in a N-cadherin dependent fashion.
- Inhibition of NF-κB results in reversal of heightened invasiveness due to N-cadherin expression.
- N-cadherin expression reduces AR expression and the expression of these proteins is inversely correlated.
- N-cadherin expression induces activation of AKT.
- Inhibition of PI3K/AKT in N-cadherin expressing cells results in specific reduction in NF-κB activation.
- N-cadherin activates JNK/AP1.
- JNK/AP1mediates invasiveness and induction of EMT-regulated transcription factors (e.g. Twist) that are stimulated by N-cadherin.
- AP1 complexes composed of c-Jun and c-Fos heterodimers bind to the upstream regulatory elements in the 5' UTR of the *Twist* gene.
- TAK1 is the MAP3K that activates both the NF-κB and JNK pathways.
- MKK4 is the MAP2K that activates JNK in a N-cadherin dependent fashion.

REPORTABLE OUTOMCES:

- Generation of inducible N-cadherin shRNA cell lines in LNCaP-C2 and PC3 background.
- Manuscript published in *Nature Medicine*.

CONCLUSIONS:

Our findings establish N-cadherin as a critical target for potential therapeutic intervention. N-cadherin inhibition blocks growth, invasion, metastasis, and castration resistance in prostate cancer models. We have documented the increase in N-cadherin expression during the progression of disease in patients. Thus, our findings have clear translational implications for the potential management of advanced prostate cancer.

N-cadherin expression induces invasiveness and EMT. This is mediated, at least in part, by downstream activation of the JNK/AP1 pathway. Specifically, N-cadherin expression induces activation of the MAP kinase, JNK, which in turn phosphorylates and activates c-Jun. Next, c-Jun forms a heterodimer with c-Fos to generate a competent AP1 transcriptional complex that induces the expression of a large cohort of genes, including *Twist*. These findings have important implications not only for the understanding of prostate cancer disease progression, but also for the development of a rational approach to drug development. For example, anti-N-cadherin antibodies could be used in conjunction with inhibitors of JNK. Moreover, inhibitors of NF-κB, including inhibitors of the kinase IKK, can be employed in combination therapy approaches with N-cadherin antibodies. Future directions will focus on co-targeting N-cadherin and other biochemical signaling pathways downstream of N-cadherin that cooperate with N-cadherin to drive invasion and tumorigenesis. As such, we will bring our model systems *in vivo* and exploit many of the compounds that are available to target these pathways. For example, JNK or IKK inhibitors can be used in conjunction with N-cadherin antibodies to effectively treat CRPC. In addition, we are identifying the biochemical pathways that link N-cadherin expression to JNK activation.

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APPENDICES:

Nature Medicine manuscript and supplementary information.

SUPPORTING DATA:

See attached figures and figure legends.

nature medicine

Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance

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The transition from androgen-dependent to castration-resistant prostate cancer (CRPC) is a lethal event of uncertain molecular etiology. Comparing gene expression in isogenic androgen-dependent and CRPC xenografts, we found a reproducible increase in N-cadherin expression, which was also elevated in primary and metastatic tumors of individuals with CRPC. Ectopic expression of N-cadherin in nonmetastatic, androgen-dependent prostate cancer models caused castration resistance, invasion and metastasis. Monoclonal antibodies against the ectodomain of N-cadherin reduced proliferation, adhesion and invasion of prostate cancer cells *in vitro*. *In vivo*, these antibodies slowed the growth of multiple established CRPC xenografts, blocked local invasion and metastasis and, at higher doses, led to complete regression. N-cadherin–specific antibodies markedly delayed the time to emergence of castration resistance, markedly affected tumor histology and angiogenesis, and reduced both AKT serine-threonine kinase activity and serum interleukin-8 (IL-8) secretion. These data indicate that N-cadherin is a major cause of both prostate cancer metastasis and castration resistance. Therapeutic targeting of this factor with monoclonal antibodies may have considerable clinical benefit.

Men with prostate cancer die predominantly from metastatic disease that is resistant to androgen deprivation therapy. Although the complete cause of castration resistance is not known, recent studies indicate that a large percentage of castration-resistant tumors progress by maintaining androgen receptor–dependent signaling. Mechanisms underlying the preservation of androgen receptor signaling include androgen receptor overexpression, growth factor–regulated androgen receptor activation and *de novo* intracrine androgen production[1–4]. New treatments designed to block androgen receptor activity (MDV3100) and steroidal synthesis (for example, abiraterone or TAK-700) have entered the clinic with promising preliminary results.

Despite these advances, it is not certain that androgen receptor reactivation is the only cause of castration resistance or that abrogation of androgen receptor signaling will result in cure. Lethal prostate cancers are heterogeneous, with pockets of cells that overexpress androgen receptor and others that do not express detectable androgen receptor[5,6]. Initial results with the newest androgen receptor–targeted drugs are extremely promising, but early data suggest that 30% of patients do not respond at all, and 30–40% have only partial responses[7,8]. The mechanisms by which tumors resist newer antiandrogens are not known, but the existence of tumors that are resistant to these approaches suggests that some tumors may be androgen receptor independent or only partially androgen receptor dependent.

There are a number of potential androgen receptor–independent mechanisms of castration resistance. For example, castration induces multiple antiapoptotic genes[9,10]. Recent clinical studies of agents that block these pathways have had initial promise. There has also been a surge of interest in the role of prostate cancer stem cells in prostate cancer development and progression[11,12]. Although controversial, some studies suggest that normal and prostate cancer stem cells may not express androgen receptor, implying that prostate cancers may become castration resistant through survival and expansion of cancer-initiating cells that lack functional androgen receptor.

To identify alternative pathways of castration resistance, we compared gene expression in matched androgen-dependent and CRPC xenografts. N-cadherin, a mesenchymal cadherin associated with epithelial-to-mesenchymal transition (EMT), was reproducibly upregulated in several models of castration-resistant cancer. We validated the association of N-cadherin with castration resistance in clinical samples of CRPC. These findings prompted us to perform a series

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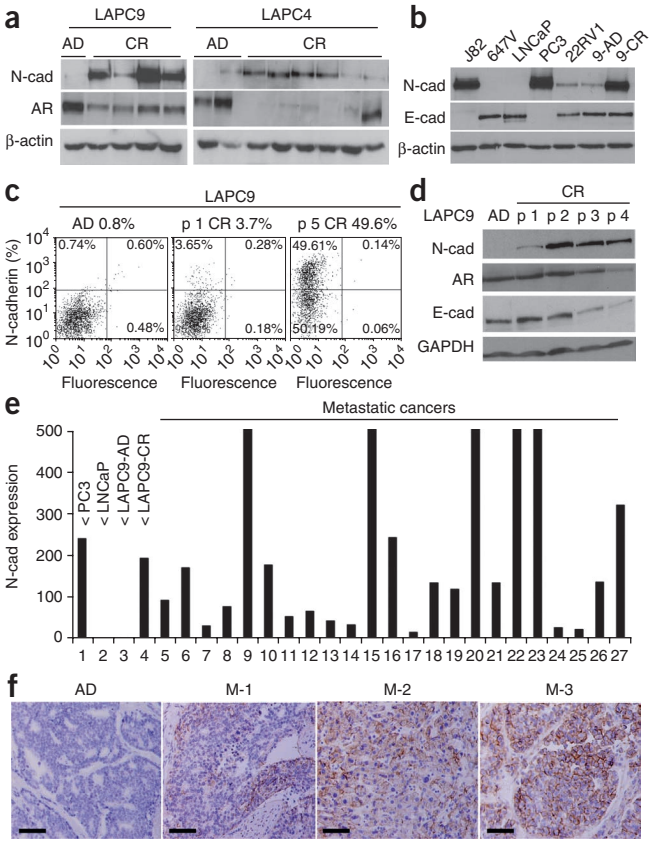

Figure 1 N-cadherin is upregulated in castration resistant prostate cancer. (**a**) N-cadherin and androgen receptor expression in multiple independently derived paired AD and CR LAPC4 and LAPC9 xenografts. (**b**) Protein expression of N-cadherin and E-cadherin in prostate cancer cells lines (LNCaP, PC3, 22RV1, LAPC9-AD and LAPC9-CR) and control cells (bladder cancer cell lines J82 and 647V). (**c**) FACS analysis of N-cadherin in serial passages (p) of LAPC9 from AD to CR. (**d**) Protein expression of N-cadherin, E-cadherin and AR in serial passages of LAPC9 from AD to CR. (**e**) Real-time PCR analysis of N-cadherin expression in multiple prostate cancer metastases (9, 15, 20, 22 and 23 are higher by more than 1,500-fold). Normalized expression (against glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) is shown as fold-change of LNCaP expression, with PC3 and LAPC9 included for comparison. (**f**) N-cadherin immunohistochemistry of high-expression prostate cancer metastases (M), showing clear staining in M1, M2 and M3 and no staining in AD. Scale bar, 500 μm.

of *in vitro* and *in vivo* studies, with the hypothesis that N-cadherin is crucial in prostate cancer progression not only to metastasis, but also to castration resistance. Because N-cadherin is expressed on the cell surface, we also asked whether therapeutic targeting with N-cadherin–specific monoclonal antibodies would have efficacy in preclinical models. The major findings of our study are that N-cadherin expression is sufficient to cause invasive, metastatic and castration-resistant prostate cancer and that these effects can be inhibited by N-cadherin–specific antibodies. Furthermore, N-cadherin–specific antibodies can inhibit the growth of both androgen receptor–positive and androgen receptor–negative prostate cancers. These studies identify a previously unknown pathway responsible for metastasis and castration resistance and validate N-cadherin as a promising new target for prostate cancer treatment.

RESULTS

N-cadherin is upregulated in CRPC

To identify markers of castration resistance, we compared gene expression in paired hormone-sensitive (AD) and castration-resistant (CR) LAPC9 xenografts[13]. N-cadherin expression was highly elevated in LAPC9-CR xenografts[13], which we confirmed by further screening of independently derived LAPC4 and LAPC9 xenografts (**Fig. 1a**). N-cadherin was absent in hormone-sensitive LNCaP but present in castration-resistant 22RV1, PC3 and LNCaP-CL1[14] prostate cancer cell lines (**Fig. 1b**). These data suggest that expression of N-cadherin is a common event in CRPC progression.

Next, we evaluated the kinetics of N-cadherin expression in serial passages of LAPC9-CR tumors in castrated mice. We detected N-cadherin in 1–5% of cells in tumors after the first passage, but it was present in 50% of cells by passage 5 (**Fig. 1c**), concomitant with gradual loss of E-cadherin and androgen receptor expression (**Fig. 1d**). These results suggest that N-cadherin–positive cells may have a growth advantage over N-cadherin–negative cells in castrated mice and that N-cadherin may be involved in the modulation of E-cadherin and androgen receptor expression.

To determine whether N-cadherin is expressed in clinical CRPC, we performed quantitative PCR and immunohistochemistry on 21 soft-tissue and bone metastases obtained from men who died from prostate cancer. N-cadherin was expressed in 16 of 21 metastases (**Fig. 1e**). Immunohistochemical staining confirmed N-cadherin protein expression in cases with high N-cadherin mRNA levels (**Fig. 1f**) and in three of six additional CRPC bone metastases. We also stained three tissue microarrays containing samples from individuals with benign prostatic hyperplasia, hormone-naive prostate cancer, prostate cancer treated with 3–9 months of neoadjuvant hormone ablation, and CRPC. We detected N-cadherin expression in 16.7%, 28%, 34% and 67% of these samples, respectively. The mean percentage of cells staining positive for N-cadherin among all samples increased from 1% in benign prostatic hyperplasia to 9.5% in hormone-naive disease, 22.5% in men treated with neoadjuvant androgen deprivation and 41% in CRPC ($P < 0.01$) (**Supplementary Fig. 1**). These data demonstrate that N-cadherin expression is rare in untreated androgen-dependent prostate cancer, increases with androgen deprivation and is highest in CRPC.

N-cadherin causes invasion, metastasis and castration resistance

To evaluate the role of N-cadherin in prostate cancer, we ectopically expressed N-cadherin in multiple AD cell lines (LNCaP, MDA-PCa-2b and LAPC4). N-cadherin–positive cells appeared flattened and fibroblastic, concomitant with loss of E-cadherin and gain of vimentin, although one low-expressing LNCaP subline (C3) retained E-cadherin and did not change morphologically (**Fig. 2a**,**b**). All N-cadherin–expressing cell lines (including C3) became more invasive (**Fig. 2c**), and invasiveness correlated with N-cadherin abundance, indicative of a gene dosage effect. When implanted subcutaneously, N-cadherin–positive tumors invaded underlying muscle and spread to distant lymph nodes (**Fig. 2a**,**c**). Conversely, silencing N-cadherin in castration-resistant PC3 and CL1 cells reduced invasiveness (**Fig. 2d**). These data suggest that N-cadherin expression is sufficient to cause EMT, invasion and metastasis in prostate cancer cells.

The association of N-cadherin with CRPC suggested that it might have a role in castration resistance. Consistent with this hypothesis, N-cadherin–expressing cell lines (MDA-N and LNCaP-C1, LNCa-C2 and LNCa-C3) could proliferate in the absence of androgen *in vitro* (**Fig. 2e**). Most importantly, N-cadherin expression

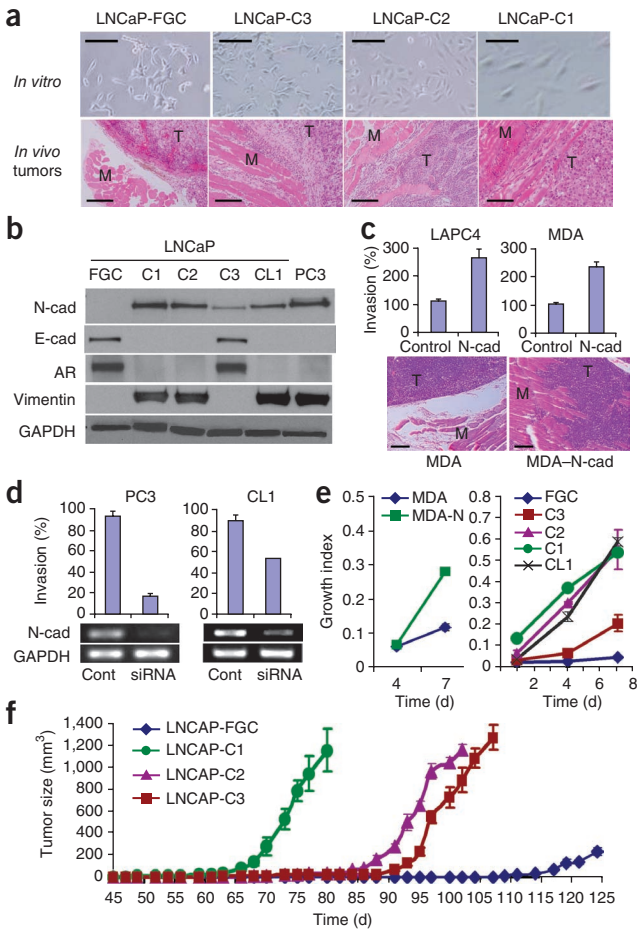

Figure 2 N-cadherin causes invasion, migration and EMT of multiple prostate cancer cell lines. (**a**) Top, *in vitro* morphologic changes in LNCaP sublines that overexpress increasing amounts of N-cadherin (LNCaP-C3 < LNCaP-C2 < LNCaP-C1) compared to control cell line LNCaP-FGC (control). Scale bar, 50 μm. Bottom, *in vivo* invasive tumor growth of LNCaP sublines in castrated mice, compared to control noninvasive tumor in intact mice. M, muscle; T, tumor. Scale bar, 500 μm. (**b**) Western blot of N-cadherin–overexpressing sublines, showing loss of E-cadherin and androgen receptor, with gain of vimentin expression in C2 and C1. CL1 and PC3 are castration-resistant cell lines with endogenous N-cadherin. (**c**) Top, invasion assays in androgen-dependent LAPC4 ($P = 0.009$) and MDA-Pca-2b ($P = 0.016$) cells ectopically overexpressing N-cadherin. Bottom, deep muscle invasion of *in vivo* MDA-N-cadherin tumor versus noninvasive MDA tumor (control). Scale bar, 100 μm. M, muscle; T, tumor. (**d**) Invasion assays in endogenous PC3 and CL1 cells upon N-cadherin silencing by siRNA silencing, $P = 0.003$. Cont, control (scrambled siRNA). (**e**) *In vitro* castration-resistant growth of both MDA-PCa-2b and LNCaP sublines overexpressing N-cadherin ($P = 0.014$ versus FGC), C3 versus FGC ($P = 0.029$). (**f**) *In vivo* castration-resistant growth of LNCaP-FGC, C1, C2, and C3 when implanted in castrated mice. Data are shown as means ± s.e.m.

conferred castration resistance *in vivo*, as evidenced by the ability of all N-cadherin–transduced cell lines to form tumors in castrated mice (**Fig. 2f**). Castration-resistant growth correlated with the level of N-cadherin expression, with LNCaP-C1 cells growing more rapidly than C2 and C3 cells *in vitro* and *in vivo* (**Fig. 2e**,**f**). To determine whether N-cadherin is required for castration resistance, we knocked it down in PC3 and LNCaP-CL1 cells. Stable silencing of N-cadherin significantly ($P = 0.005$) impaired the ability of both cell lines to form tumors in castrated mice (**Supplementary Fig. 2**). These data suggest that N-cadherin expression is both sufficient and necessary for castration resistant growth.

N-cadherin expression led to an inverse loss of androgen receptor, with high expressors (LNCaP-C1, PC3 and CL1) losing androgen receptor completely and low- expressors (LNCaP-C3) retaining it (**Fig. 1a** and **Fig. 2b**). We saw a similar pattern in LAPC9-CR cells, with some cells expressing androgen receptor, some expressing N-cadherin and others expressing both androgen receptor and N-cadherin (**Supplementary Fig. 3**). These data indicate that N-cadherin is sufficient to cause androgen receptor–independent prostate cancer. However, many prostate cancers coexpress N-cadherin and androgen receptor, suggesting that these factors may act synergistically to promote castration-resistant growth.

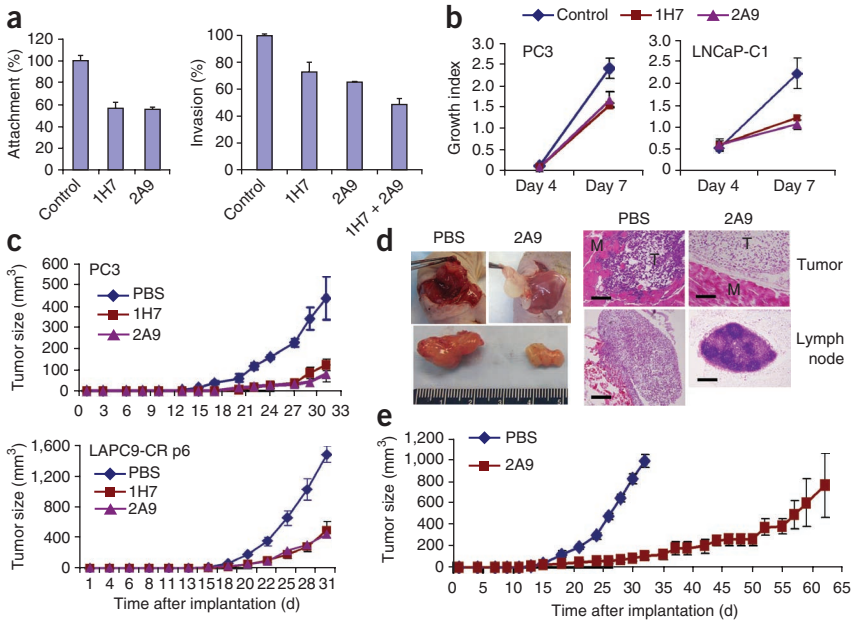

Figure 3 Antibodies against N-cadherin decrease invasion and tumor growth. (**a**) Attachment ($P = 0.004$) and invasion ($P = 0.05$) assays of PC3 cells upon treatment without or with 1H7 or 2A9 (80 μg ml^{-1}). (**b**) Decrease in cell growth measured by proliferation assays of PC3 ($P = 0.014$) and LNCaP-C1 ($P = 0.01$) cells, upon treatment without or with 80 μg ml^{-1} 1H7 or 2A9. (**c**) *In vivo* castration-resistant growth inhibition (>70%) of both PC3 and LAPC9-CR (passage 6) tumors upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight, beginning when subcutaneous tumors were palpable in castrated mice. $P = 0.016$ for both cell lines compared to control (PBS) group at 31 d. (**d**) Gross and histological analyses of mice treated without or with 2A9, showing decrease in tumor size, tumor-muscle invasion (scale bar, 200 μm) and metastases to axillary lymph nodes (scale bar, 500 μm). (**e**) Continuous growth inhibition of PC3 tumors upon long-term treatment with 2A9 antibody (50% at 57 d compared to control group at 32 days, $P = 0.025$). Data are shown as means ± s.e.m.

N-cadherin antibodies inhibit growth of CRPC

The presence of N-cadherin in metastatic prostate cancer and its ability to promote castration resistance suggested that N-cadherin might be a therapeutic target in advanced prostate cancer. We generated a panel of monoclonal antibodies specific for the extracellular domain of N-cadherin to test this hypothesis and to determine which domains are necessary for its effects in prostate cancer. We screened antibodies for cell surface recognition of N-cadherin and an ability to inhibit invasion *in vitro*. We selected two antibodies: 1H7, a mouse IgG1, recognizes an epitope within the first three extracellular domains, whereas 2A9, an IgG2a, recognizes an epitope in the fourth domain. Both antibodies inhibited invasion, attachment and proliferation of PC3 and LNCaP-C1 cells *in vitro* (**Fig. 3a**,**b**). Upon exposure to either antibody, PC3 and LNCaP-C1 cells showed morphologic changes, such as increased polarity, resembling an epithelial phenotype (data not shown). These results suggest that N-cadherin–specific antibodies can affect multiple parameters of *in vitro* growth, including invasion, proliferation, attachment and potentially EMT.

We next asked whether N-cadherin–specific antibodies could affect invasion, metastasis and castration-resistant tumor growth *in vivo*. Castrated mice bearing palpable PC3, LAPC9-CR and LNCaP-C1 tumors were treated twice weekly with PBS or the antibodies 1H7 or 2A9 (10 mg per kg body weight) for 2 weeks. Both antibodies inhibited tumor growth (**Fig. 3c**). The antibody-treated tumors were pale, nonadherent to underlying muscle, and noninvasive histologically, whereas control tumors grossly invaded underlying muscle (**Fig. 3d**). In addition, N-cadherin–specific antibody–treated mice had rare distant lymph node metastases (one out of five mice treated with 1H7, zero of five mice treated with 2A9), whereas 100% of nodes (five of five) were replaced by cancer in control mice (**Fig. 3d**). Prolonged administration of 2A9 led to long-term growth suppression and a >100% mean improvement in survival of mice bearing PC3 tumors (**Fig. 3e**). Treated tumors had large areas of cell loss, reduced proliferation (Ki-67 staining), fewer blood vessels (CD31 staining), less vimentin staining and lower N-cadherin expression compared to untreated tumors (**Fig. 4** and **Supplementary Fig. 4**).These data indicate that antibodies targeting the N-cadherin ectodomain are able to inhibit tumor growth, local invasion and metastasis of CRPC.

We also administered N-cadherin–specific antibodies to mice with larger established tumors. Both antibodies significantly slowed the growth of all three tumor models, although 2A9 suppressed growth better than 1H7 in most experiments (**Fig. 5a**). Dose escalation of 2A9 to 20 mg per kg body weight led to complete regression of >50% of PC3 tumors, whereas no additional benefit was seen with 40 mg per kg body weight (**Fig. 5b**). To examine the mechanism of tumor regression, we collected a subset of tumors within days of starting antibody treatment (**Fig. 5c**). We saw large areas of cell loss and necrosis in treated tumors, as well as more caspase-3 staining (**Fig. 5d**), suggesting that apoptosis may temporally precede the cell loss seen after prolonged treatment. These data show that N-cadherin–specific antibodies can suppress the growth of large established tumors and that higher doses can cause tumor regression.

N-cadherin antibodies delay progression to castration resistance

To test the requirement for N-cadherin in castration-resistant progression, we implanted LAPC9-AD tumors into castrated mice, treated them with N-cadherin–specific antibodies and monitored time to castration-resistant growth. Treatment with 2A9 significantly delayed time to castration resistance, whereas 1H7 only briefly delayed tumor

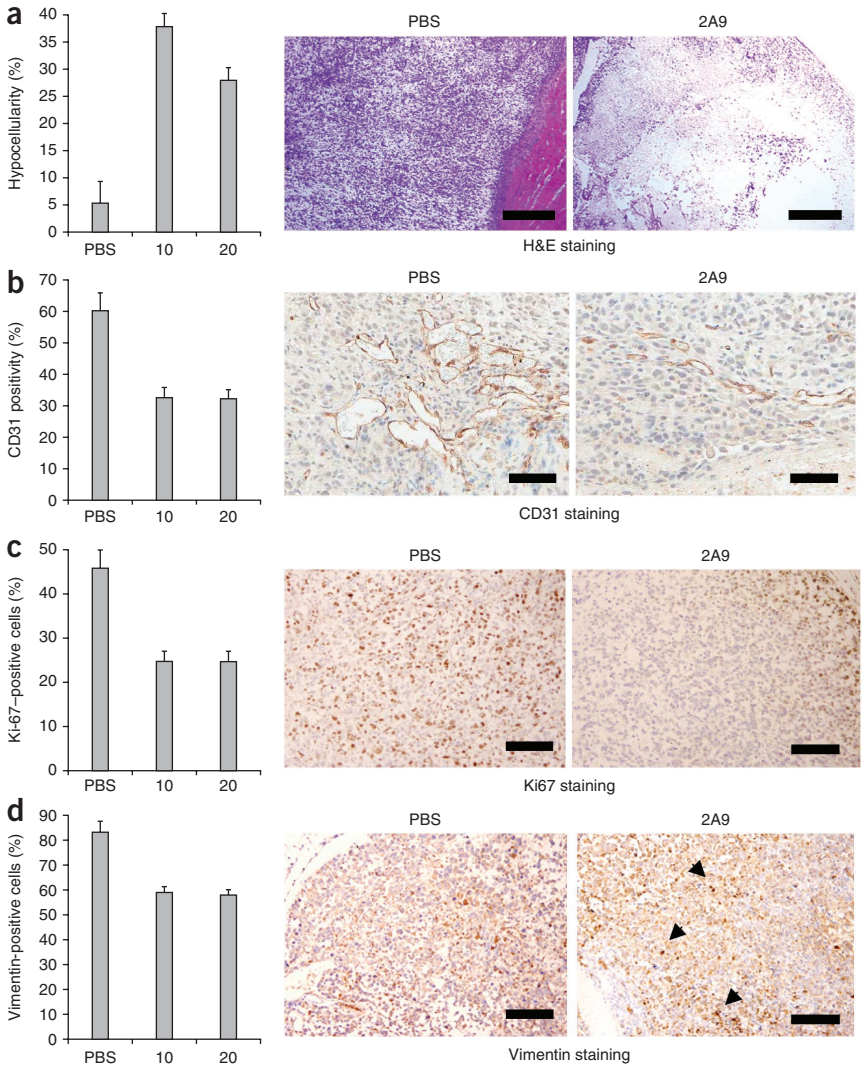

Figure 4 Histological and immunohistochemical assessments of PC3 tumors treated without or with 2A9 at 10 or 20 mg per kg body weight. (**a**–**d**) Quantification of changes between untreated and treated tumors in the following parameters: hypocellular regions ($P = 0.004$) by H&E staining (scale bar, 1.0 mm) (**a**); CD31 staining ($P < 0.001$; scale bar, 500 µm) (**b**); Ki-67 staining ($P = 0.002$; scale bar, 500 µm) (**c**); vimentin-positive regions (20 mg per kg body weight dose, $P = 0.032$) (**d**). The arrowhead points to stained cells. Scale bar, 500 µm. Quantification was determined by counting five different fields per tumor, followed by averaging the values for the five tumors. Data are shown as means ± s.e.m.

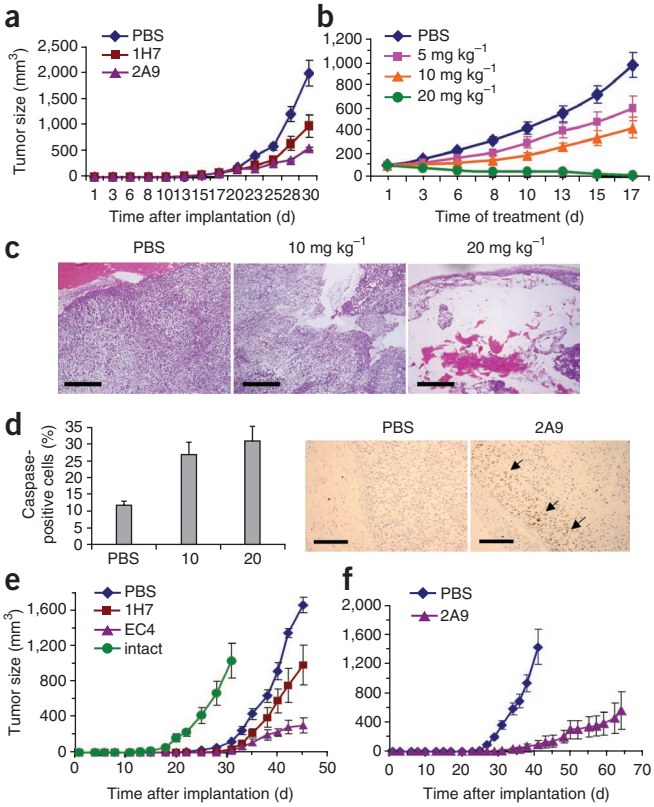

Figure 5 N-cadherin antibodies inhibit growth of established tumor and block progression to castration resistance *in vivo*. (**a**) Growth inhibition of established LAPC9-CR tumors (100 mm^3) upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight. $P = 0.003$ for both antibodies compared to control (PBS) group at 30 d. (**b**) Growth inhibition of established PC3 tumors (100 mm^3) upon treatment without or with escalating doses of 2A9, starting at 5 mg per kg body weight. $P = 0.024$ compared to control (PBS) group at 17 d. (**c**) Histology of untreated versus antibody-treated tumors. Scale bar, 1.0 mm. (**d**) Caspase-3 staining in untreated versus antibody-treated tumors at both 10 and 20 mg per kg body weight doses ($P < 0.005$). Arrow, stained cells. Scale bar, 500 μm. (**e**) Delay of LAPC9-CR tumor emergence upon treatment without or with 1H7 or 2A9 at 10 mg per kg body weight. $P = 0.023$ compared to control (PBS) group at 45 d. Intact, mice bearing LAPC9-AD tumors without castration. (**f**) Same experiment as in **e** but with continuous 2A9 treatment, showing prolonged suppression of CR tumor growth after progression to castration resistance. Data are shown as means ± s.e.m.

growth (**Fig. 5e**,**f**). Of note, only ~4% of cells in the untreated control tumors expressed N-cadherin, consistent with previous experiments showing that N-cadherin is expressed by a minority of cells in early-passage CR tumors (**Fig. 1c**). These data suggest that N-cadherin is required for the development of castration resistance and that therapeutic targeting of N-cadherin in AD tumors (even in a minority of cells) can markedly delay the emergence of CRPC.

N-cadherin alters expression of genes implicated in CRPC

To gain insight into the mechanism of N-cadherin activity in prostate cancer, we compared the expression profiles of N-cadherin–transduced cells and controls. We selected genes previously shown to be associated with progression of LNCaP cells to the castration-resistant LNCaP-CL1 subline as a starting point[14]. As predicted, N-cadherin–transduced cells showed the characteristic changes of an EMT, with decreased E-cadherin expression (**Fig. 6a**) and increased vimentin expression (data not shown). These changes were proportional to the level of N-cadherin expression. Other notable changes included increased B cell lymphoma-2 (bcl-2) expression (data not shown), increased transforming growth factor-β1 (TGF-β1), TGF-β2 and vascular endothelial growth factor (VEGF) expression, reduced androgen receptor and prostate-specific antigen expression and increased IL-6 and IL-8 expression (**Fig. 6a**). The loss of androgen receptor is consistent with our observation in LAPC-9 cells that N-cadherin expression is inversely correlated with androgen receptor expression. Bcl-2 expression may explain the ability of N-cadherin–positive cells to survive in an androgen-depleted environment[15]. TGF-β can induce EMT and might mediate N-cadherin signal transduction. TGF-β, IL-6 and IL-8 have all previously been implicated in CRPC[16,17]. Silencing of N-cadherin in PC3 cells decreased IL-6, IL-8, vimentin, TGF-β and VEGF expression but did not restore androgen receptor or E-cadherin expression, suggesting that more prolonged knockdown might be required for complete reversal of EMT (**Supplementary Fig. 5**).

Previous studies have associated N-cadherin with phosphoinositide 3-kinase–AKT pathway activation[15]. N-cadherin silencing reduced AKT phosphorylation, whereas N-cadherin overexpression correlated with increased AKT activity (**Fig. 6b**,**c**). These results indicate that N-cadherin is sufficient to cause EMT and regulates the expression of multiple genes implicated in castration resistance.

N-cadherinantibody reduces AKT activity and IL-8 secretion

To determine the effects of N-cadherin–targeting antibody treatment on gene expression, we exposed PC3 and LNCaP-C2 cells *in vitro* to 2A9 and determined whether 2A9 reduced AKT kinase activity and IL-8 production. 2A9 reduced both phospho-AKT abundance and AKT kinase activity over a 4- to 24-h time period (**Fig. 6d**). ELISA of cell culture media after 2A9 treatment showed a >50% reduction

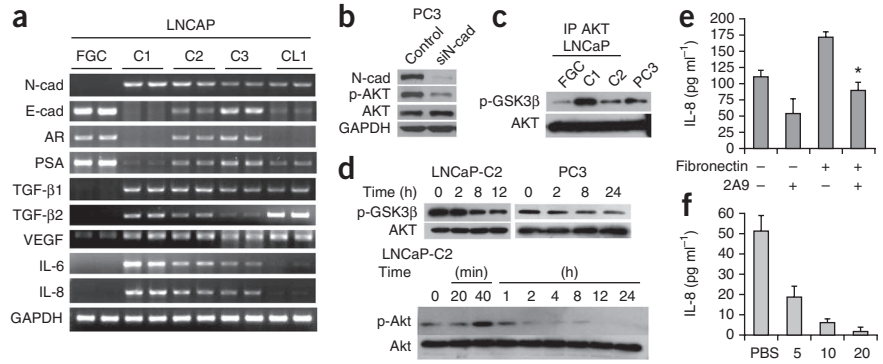

Figure 6 Gene expression change in N-cadherin–overexpressing cells. (**a**) RT-PCR analyses of gene expression in LNCaP cells without (FGC) or with high (C1, C2 and CL-AI) and low (C3) levels of N-cadherin. (**b**) Western blot of PC3 cells upon N-cadherin siRNA silencing (siN-cad). (**c**) AKT kinase activity in N-cadherin–overexpressing cell lines, measured by *in vitro* kinase assay. (**d**) Changes in AKT kinase activity and phospho-AKT level in *in vitro* time-course treatment with 2A9 at 80 μg ml^{-1} in LNCaP-C2 or PC3 cells. (**e**) Changes in fibronectin-induced IL-8 secretion in cell media (*$P = 0.027$) upon *in vitro* 2A9 treatment at 80 μg ml^{-1} in LNCaP-C1. (**f**) Changes in serum IL-8 level in PC3 tumor–bearing mice treated with 2A9 antibody at 5 ($P = 0.014$), 10 and 20 mg per kg body weight ($P < 0.001$). Data are shown as means ± s.e.m.

in IL-8 secretion (**Fig. 6e**). 2A9 treatment also led to progressive declines in serum IL-8 that correlated with antibody dose and tumor regression (**Fig. 6f**). These data indicate that the N-cadherin–specific antibody 2A9 can reverse N-cadherin–induced activation of AKT and IL-8 expression and may explain, at least in part, the antitumor activity of this antibody. IL-8 could serve as a potential biomarker of N-cadherin and N-cadherin–targeted therapy.

DISCUSSION

N-cadherin expression is reproducibly associated with progression to castration resistance in both LAPC4 and LAPC9 prostate cancer xenografts. N-cadherin is expressed in multiple CRPC cell lines and in a majority of metastatic and castration-resistant prostate cancer tissues. N-cadherin induction after neoadjuvant hormone ablation supports the association of this protein with castration resistance. Our findings differ somewhat from previous studies that have reported higher N-cadherin expression in high-risk primary tumors. For example, one group reported that N-cadherin was expressed in 50% of high-grade primary tumors and lymph node metastases[18] and in 65% of tumors with Gleason score of ≥ 7 (ref. 19). Another study showed that an E- to N-cadherin switch in primary tumors was predictive of recurrence and prostate cancer–related death[20]. Some of the differences between our results and those of these studies might be ascribed to technical issues such as antibody selection. Differences in the subject populations (that is, Europe versus US) might also explain the differences in reported expression between the studies. Regardless, our study and others confirm that N-cadherin is expressed in a considerable percentage of human prostate cancers and validate N-cadherin as a promising therapeutic target in this disease.

N-cadherin expression increases with passaging of castration-resistant tumors in our xenograft models, suggesting that N-cadherin–positive cells have a growth advantage over N-cadherin–negative cells and that a small percentage of N-cadherin–positive cells may be sufficient to drive castration resistance. Consistent with these hypotheses, N-cadherin–positive cells proliferate more rapidly than N-cadherin–negative cells. N-cadherin–positive cells from LAPC9-CR tumors are also more tumorigenic than N-cadherin–negative cells (E.K. and R.E.R., unpublished data). A number of recent studies have linked EMT and EMT-associated genes with cancer stem cells. Induction of EMT in immortalized mammary cells produced cells with stem cell properties such as mammosphere formation and tumorigenicity[21]. These studies raise the possibility that N-cadherin may be a marker for a population of castration-resistant stem cells in prostate cancer. This possibility is supported by our finding that N-cadherin–positive cells are tumorigenic and that antibody treatment was sufficient to delay progression to castration resistance, even though N-cadherin was only expressed by a small percentage of cells in the untreated controls. It is also supported by our finding that N-cadherin is expressed by only a fraction of cells in many human primary tumors, and this expression increases after androgen ablation and recurrence. Additionally, many stem cell–associated genes are upregulated in N-cadherin–positive cells (**Supplementary Fig. 6**)[22]. Additional studies will be required to establish whether N-cadherin–expressing cells are prostate cancer stem cells and whether they are required for castration-resistant or metastatic progression. Nevertheless, our data suggest that targeting of a small subset of cells with the potential to initiate castration-resistant tumor growth may be sufficient to have a therapeutic impact on this disease.

N-cadherin expression was associated with a loss or reduction in androgen receptor expression. N-cadherin–positive tumors expressed lower levels of androgen receptor than androgen-dependent control tumors, and double-staining of LAPC9-CR tumors confirmed that androgen receptor was absent in a subset of N-cadherin–positive tumor cells. Forced N-cadherin expression resulted in androgen receptor loss proportional to the level of N-cadherin expression in LNCaP sublines. The mechanism by which N-cadherin reduces androgen receptor expression is not known. Additional studies will be required both to confirm this inverse correlation and to elucidate the pathway by which N-cadherin regulates androgen receptor. However, the major implication of our data is that N-cadherin may be a cause of androgen receptor–independent prostate cancer or may synergize with low-level androgen receptor expression. It will be crucial to determine whether N-cadherin can cause resistance to newer androgen receptor–targeted therapies.

The major findings of this paper are that N-cadherin can cause castration resistance and that therapeutic targeting of N-cadherin can delay CRPC progression. The mechanisms by which N-cadherin causes castration resistance, and by which N-cadherin-targeting antibodies inhibit it, are not known. However, N-cadherin activates gene encoding proteins previously implicated in castration resistance, such as IL-8, IL-6, TGF-β, phosphoinositide 3-kinase and AKT, and bcl-2. For example, IL-8 is sufficient to cause castration resistance in androgen-dependent LNCaP and LAPC4 cells[16]. It has been shown that introduction of IL-8 leads to a decrease or loss in androgen receptor expression[16], similar to what we saw with N-cadherin. N-cadherin–specific antibody 2A9 may act in part by reducing IL-8 secretion. Alternatively, the decrease in IL-8 could reflect the reduction in tumor volume caused by 2A9. One possible practical application of this observation would be to use IL-8 as a surrogate marker of antibody activity in future clinical trials. AKT has also been implicated in CRPC[23]. N-cadherin upregulated AKT activity, and exposure of PC-3 and LNCaP-C1 cells to 2A9 reduced this activity, even in PTEN-null cell lines. These data suggest that inhibition of N-cadherin–regulated AKT activation might be another mechanism by which 2A9 exerts its antitumor effect.

It is not clear why antibody 2A9 is superior to antibody 1H7 in some experiments. Although both antibodies could block invasion and metastasis, only 2A9 could reliably affect the growth of larger tumors or substantially delay progression to castration resistance. One possibility is that the epitope on the fourth extracellular domain recognized by 2A9 is essential for N-cadherin signaling, particularly in castration-resistant growth[24]. Alternatively, the differential activity could be related to differences in affinity or immune activation (antibody-dependent cellular cytotoxicity or complement-dependent cytotoxicity). Additional work will be required to understand the roles of the antibodies or the epitopes they recognize.

The finding that N-cadherin–targeted antibodies delay castration-resistant progression and inhibit growth, invasion and metastasis raises the possibility that these antibodies may be translatable to the clinic. Their toxicity is one question that needs to be addressed, as N-cadherin is expressed broadly in normal tissues such as peripheral nerve, heart and liver. Loss of N-cadherin can disrupt the intercalated disc structure in the heart, leading to ventricular tachycardia and sudden death in conditional-knockout mice[25]. Because 1H7 cross-reacts with mouse and human N-cadherin, we checked mice treated with 1H7 for signs of cardiac or other distress. Even at doses of 40 mg per kg body weight, we saw no evidence of toxicity, with no cases of sudden death, histologic heart abnormalities or changes in serum cardiac enzymes. These results suggest that therapeutic targeting of N-cadherin may be safe, although further preclinical and clinical testing will be required to confirm the safety of this approach.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

H.T. and E.K. designed and conducted *in vitro* and *in vivo* studies. C.P.T. generated stable N-cadherin–knockdown reagents and prepared the manuscript. H.M. made the N-cadherin–overexpressing cell lines. J.Y. and R.W. performed gene and protein expression analyses. T.S. contributed to the *in vivo* N-cadherin–knockdown and antibody studies. F.L. and M.G. conducted immunohistochemical evaluation of prostate cancer specimens. J.H. contributed to immunohistochemical analyses of *in vivo* studies. R.L.V. provided clinical materials for the initial N-cadherin screening in metastases. J.A. and M.B.R. provided data on AKT activity. S.H. performed gene expression analysis for stem cell markers. Z.A.W. generated the monoclonal antibodies. R.E.R. conceived of the study and supervised the project. All authors discussed the results and commented on the manuscript at all stages.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Cell culture. LNCaP-FGC, MDA-PCa-2b and PC-3 cells were from the American Type Culture Collection and cultured as specified. LNCaP-CL1 cells[14] were provided by C.L. Tso and maintained in phenol-red–free RPMI-1640 with 10% charcoal stripped serum (DCC-FBS). LAPC4 and LAPC9 xenografts were passaged in male severe combined immunodeficient (SCID) mice[26] (Taconic).

Antibodies. Western blot analysis was performed as previously described[27] with antibodies specific for N-cadherin (3B9 from Invitrogen, clone 32 from BD Transduction Laboratories), E-cadherin (Zymed Laboratories), vimentin (Thermo Scientific), androgen receptor, glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology), AKT and phospho-AKT (Cell Signaling Technology). Immunohistochemistry was performed as previously described[28], with antibodies specific for CD31 (Santa Cruz Biotechnology), vimentin, Ki-67 (DakoCytomation) and caspase-3 (Cell Signaling Technology). Flow cytometry was performed with N-cadherin–specific antibody GC-4 (Sigma).

Establishment of N-cadherin–overexpressing and N-cadherin–knockdown cells. Full-length N-cadherin cDNA was subcloned into the lentiviral vector CSCG (Addgene) to make CSCG–N-cadherin. The lentiviral stock was produced in 293T cells by transfecting 6.25 μg CSCG–N-cadherin, 2.5 μg envelope plasmid VSVG and 6.25 μg packaging plasmid pΔVPR (provided by I. Chen). LNCaP-FGC, MDA-PCa-2b and LAPC4 cells were transduced with either CSCG–N-cadherin or CSCG-GFP lentiviruses and sorted for positive cells. LNCaP-C1, LNCaP-C2 and LNCaP-C3 were derived through limiting dilution and screening individual clones by RT-PCR for varying levels of N-cadherin and androgen receptor expression. For stable knockdown, shRNA against N-cadherin was subcloned into the GFP-positive lentiviral vector FG-12 (Addgene) to make FG12-shNcad. Scrambled shRNA was also used to make control vector. Lentiviruses were produced as described above and used to transduce PC3 and CL1 cells. One week after transduction, FG12-shNcad–transduced cells were labeled with N-cadherin–specific antibody and sorted by flow cytometry, gating for a GFP-positive, N-cadherin^{low} population. The cell lines with control vector were not sorted but were confirmed to be >50% GFP positive. After the sort, cells were immediately implanted in castrated mice as described below (*in vivo* assays).

Purification and characterization of N-cadherin–specific monoclonal antibodies. The 1H7 (IgG1-κ) and 2A9 (IgG2a-κ) N-cadherin–specific hybridomas were raised against His-tagged N-cadherin proteins representing the first three and fourth extracellular domains as previously described[26] and screened by ELISA[13] and FACS. Hybridomas were cultured in HL-1 medium (Lonza) in Integra CL 1000 flasks following the manufacturer's instructions (IBS Integra Biosciences). 1H7 and 2A9 monoclonal antibodies were purified by protein-G affinity chromatography (GE), and BIAcore 3000 (Precision Antibody Service) analysis was done with recombinant His-tagged N-cadherin as antigen.

***In vitro* assays.** Cell proliferation was measured with the CCK-8 kit (Dojindo). For attachment assays, collected cells were pretreated with 1× PBS or 80 μg ml^{-1} 2A9 antibody at 37 °C for 2 h, plated in fibronectin-coated 96-well plates without or with 2A9 for 15 min, and washed twice with 1× PBS. Attached cells were quantified by crystal violet staining. Invasion assays were performed in 24-well Matrigel invasion chambers (BD Biosciences) as previously described[26], in the presence of PBS control or 80 μg ml^{-1} 2A9 for 48 h. For knockdown experiments, cells were first transfected with commercial N-cadherin and nontargeting (control) siRNA pools, and 24 h later plated into invasion chambers, followed by quantification of invasion 48 h later. Two different pools of N-cadherin siRNA (Santa Cruz Biotechnology and Dharmacon) were used to verify the results in both cell lines.

Interleukin-8 assay. Conditioned media or mouse sera (50 μl) were assayed for IL-8 with the Human IL-8 (CXCL8) ELISA Kit (R&D Systems). Mouse blood samples were obtained retro-orbitally, and sera were separated by centrifugation.

***In vivo* studies.** All *in vivo* experiments were performed according to approved protocols from the Animal Research Committee at the University of California–Los Angeles. PC3 and N-cadherin–expressing LNCaP cells (1 × 10^6 cells) in 50% Cultrex (Trevigen) were implanted subcutaneously in 6- to 8-week-old castrated male nude (Charles River) and SCID mice, respectively. LAPC9-CR xenograft tumors were collected from castrated male SCID mice and processed to single-cell suspensions as previously described[26]. We injected 1 × 10^6 cells subcutaneously into 6- to 8-week-old castrated male SCID mice. N-cadherin–specific antibody (500 μl) at 10 or 20 mg per kg body weight, or 1× PBS control was injected intraperitoneally twice weekly, when the tumors were palpable, 100 mm^3 in size or 200 mm^3 in size. Tumors were measured with calipers, and tumor volume was calculated as follows: (larger diameter) × (smaller diameter) × (third diameter, or width). To show the specificity of the N-cadherin–specific antibody, we did an initial experiment with N-cadherin–negative LAPC9-AD and N-cadherin–positive LAPC-9CR tumors, which showed antibody efficacy only in the positive tumors (data not shown).

For progression to castration-resistant studies, LAPC9-AD xenograft tumors were collected from intact male SCID mice, and 1 × 10^6 cells were injected subcutaneously into either intact or castrated 6- to 8-week-old male SCID mice pretreated with either PBS control or 10 mg kg^{-1} body weight N-cadherin–specific antibody. Thereafter, treatments were performed twice a week, and tumor volumes were monitored as described above.

Statistical analyses. Data are shown as means ± s.e.m. Where indicated, P values were determined by unpaired Student's t test. $P \leq 0.05$ was considered significant.

26. Klein, K.A. *et al.* Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat. Med.* **3**, 402–408 (1997).
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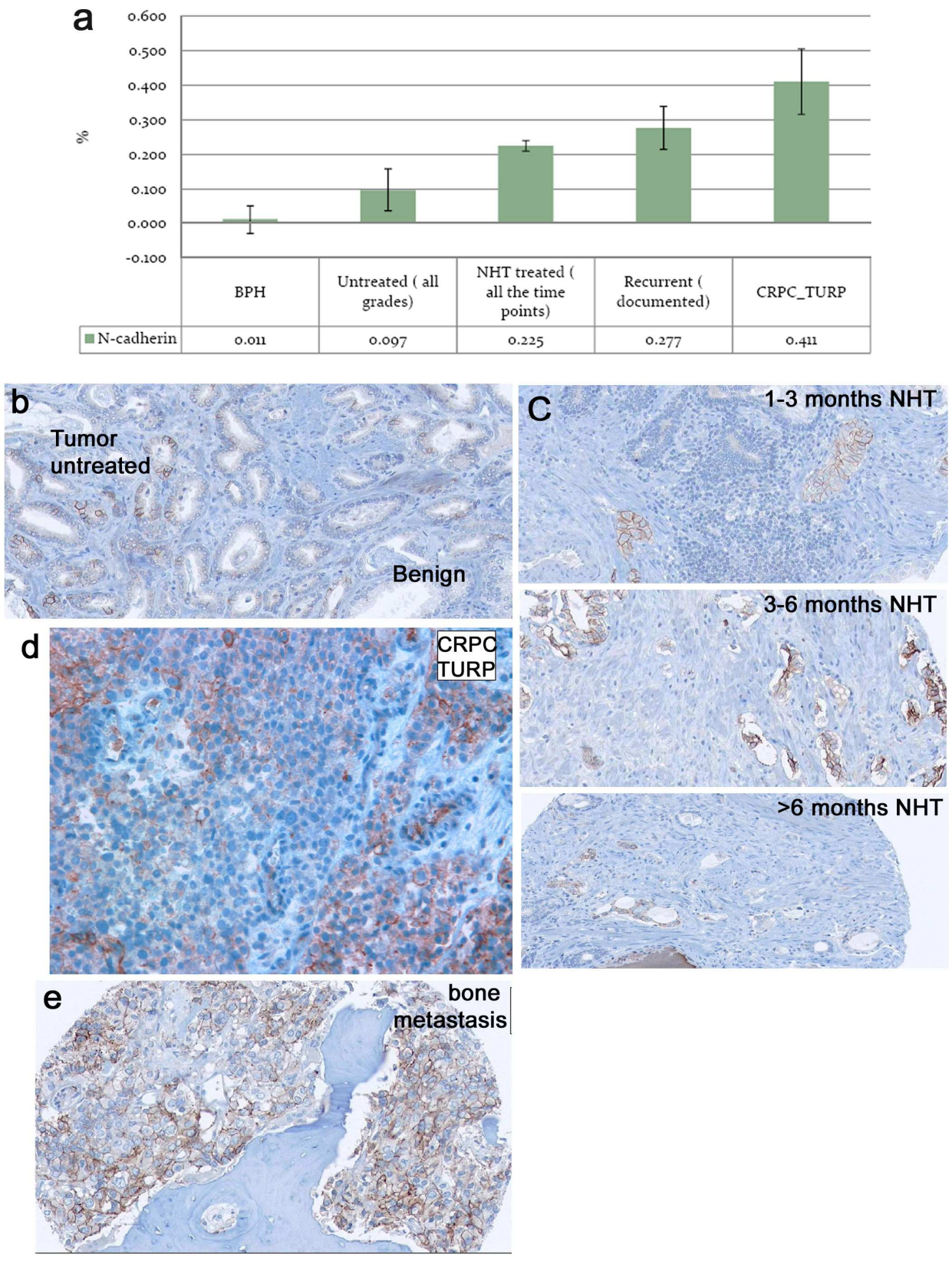
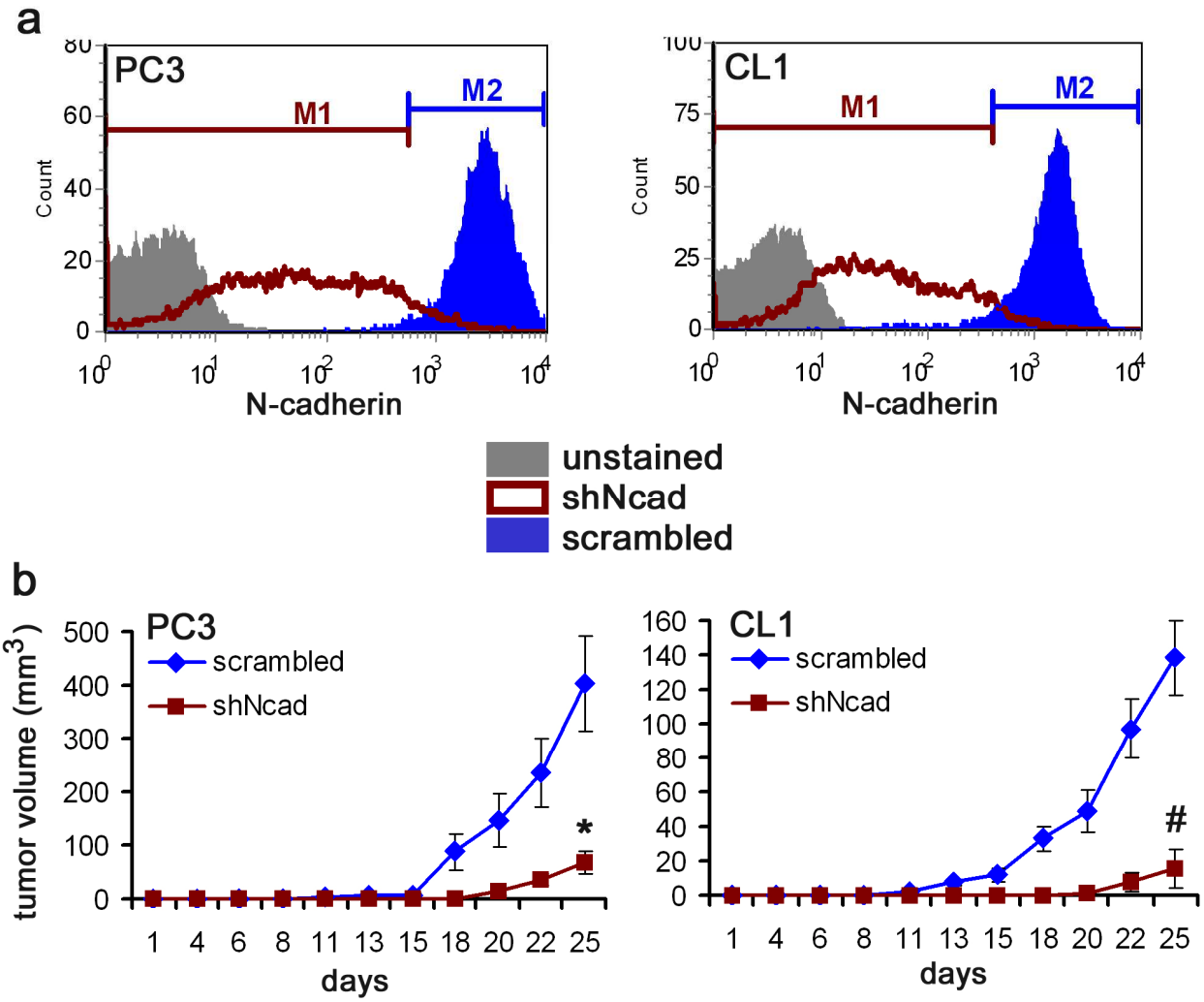
Monoclonal antibody targeting of N-cadherin inhibits prostate cancer growth, metastasis and castration resistance

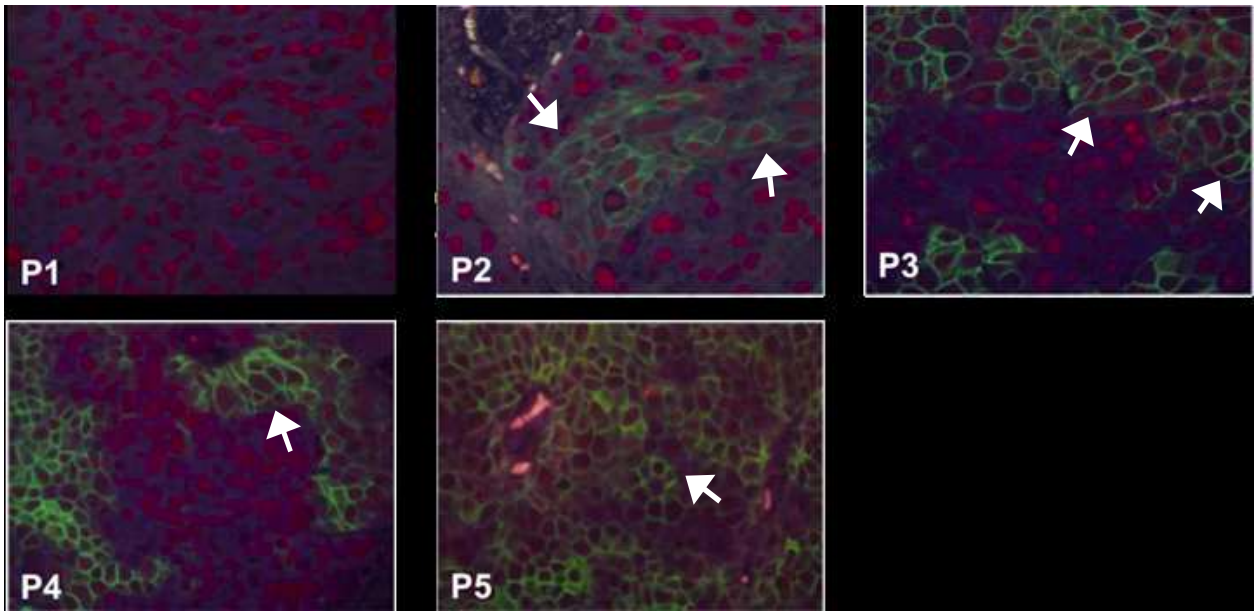
Tanaka H, Kono E, Tran CP, Miyazaki H, Yamashiro J, Shimomura T, Ladan F, Wada R, Huang J, Vessella RL, An J, Horvath S, Gleave M, Rettig MB, Wainberg ZA, Reiter RE


a
0.600
0.500
0.400
0.300
0.200
0.100
0.000
-0.100
%
BPH
Untreated (all grades)
NHT treated (all the time points)
Recurrent (documented)
CRPC_TURP
N-cadherin
0.011
0.097
0.225
0.277
0.411
b
Tumor untreated
Benign
C
1-3 months NHT
3-6 months NHT
>6 months NHT
d
CRPC TURP
e
bone metastasis

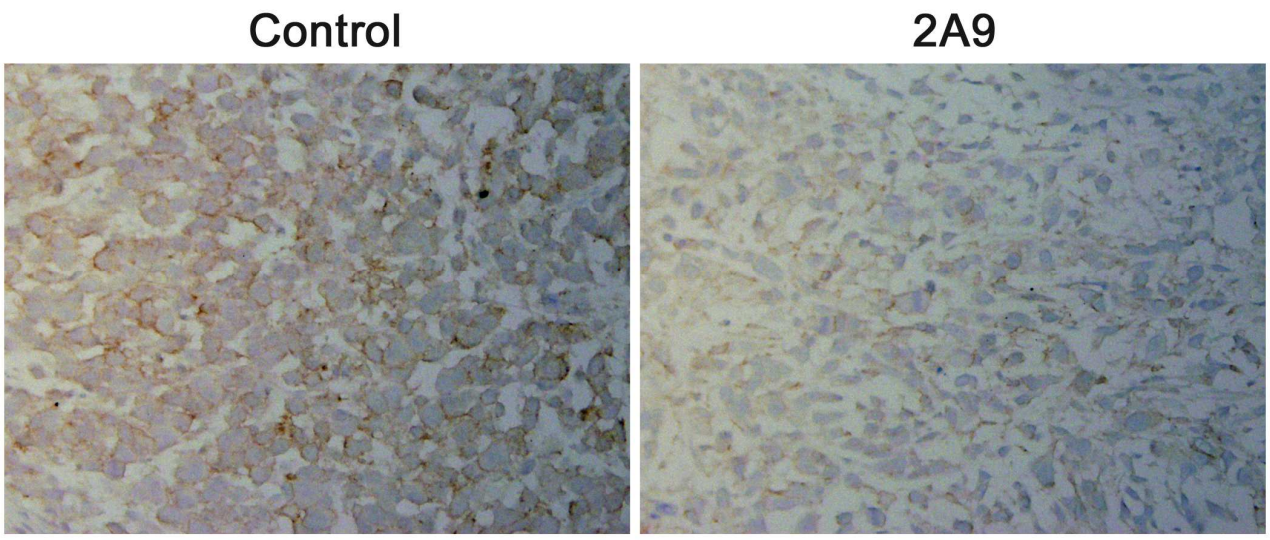
Supplementary Fig. 1. Immunohistochemical evaluation of N-cadherin in 304 prostate cancer patients with localized, hormonally treated, and castration resistant prostate cancer. All specimens were from radical prostatectomy except for 12 CRPC samples that were obtained from transurethral resection of the prostate (TURP) and 14 metastatic specimens provided by RL Vessella. Staining was performed using a mouse anti N-cadherin antibody (Clone32, BD Transduction Laboratories). **(a)** BPH: benign prostate hyperplasia; NHT: neoadjuvant hormone therapy; CRPC: castrate resistant prostate cancer. The data show mean percentage of cells staining positive for N-cadherin among all patients. The intensity of staining was found to be uniformly strong in positive cases and was therefore not included in the scoring system. **(b)** Normal epithelia were negative, while untreated tumor cells show rare immunoreactivity for N-cadherin. **(c)** N-cadherin was significantly upregulated in NHT samples, and also in **(d)** CRPC. **(e)** N-cadherin was positive in 3 out of 6 prostate cancer bone metastases. See text for additional data on the percentage of patients in each group.



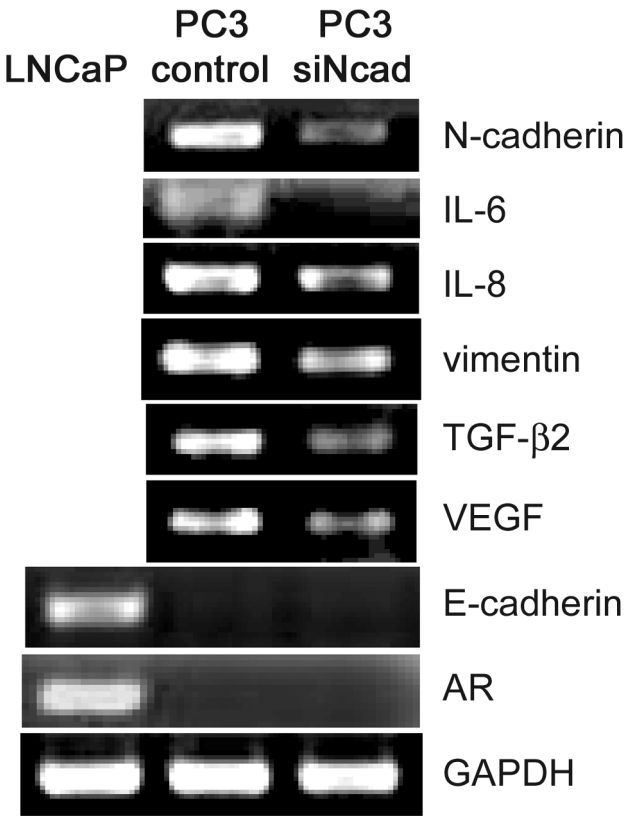
Supplementary Fig. 2. Endogenous N-cadherin shRNA knockdown (shNcad) suppressed castrate resistant growth of prostate cancer xenografts PC3 and CL1. Both cell lines were transduced with shNcad or control (scramble shRNA) GFP-lentivector, sorted for the GFP positive population, followed by subcutaneous implantation in castrated nude mice. **(a)** FACS analyses of transduced PC3 and CL1 cells 2 weeks after sorting. Note that N-cadherin positivity decreased markedly, as depicted by the peak shift from M2 to M1. **(b)** *In vivo* castrate resistant tumor growth of transduced PC3 and CL1. Significant tumor inhibition was observed in the shNcad tumor group for both xenograft models. *P = 0.005, #P = 0.001.



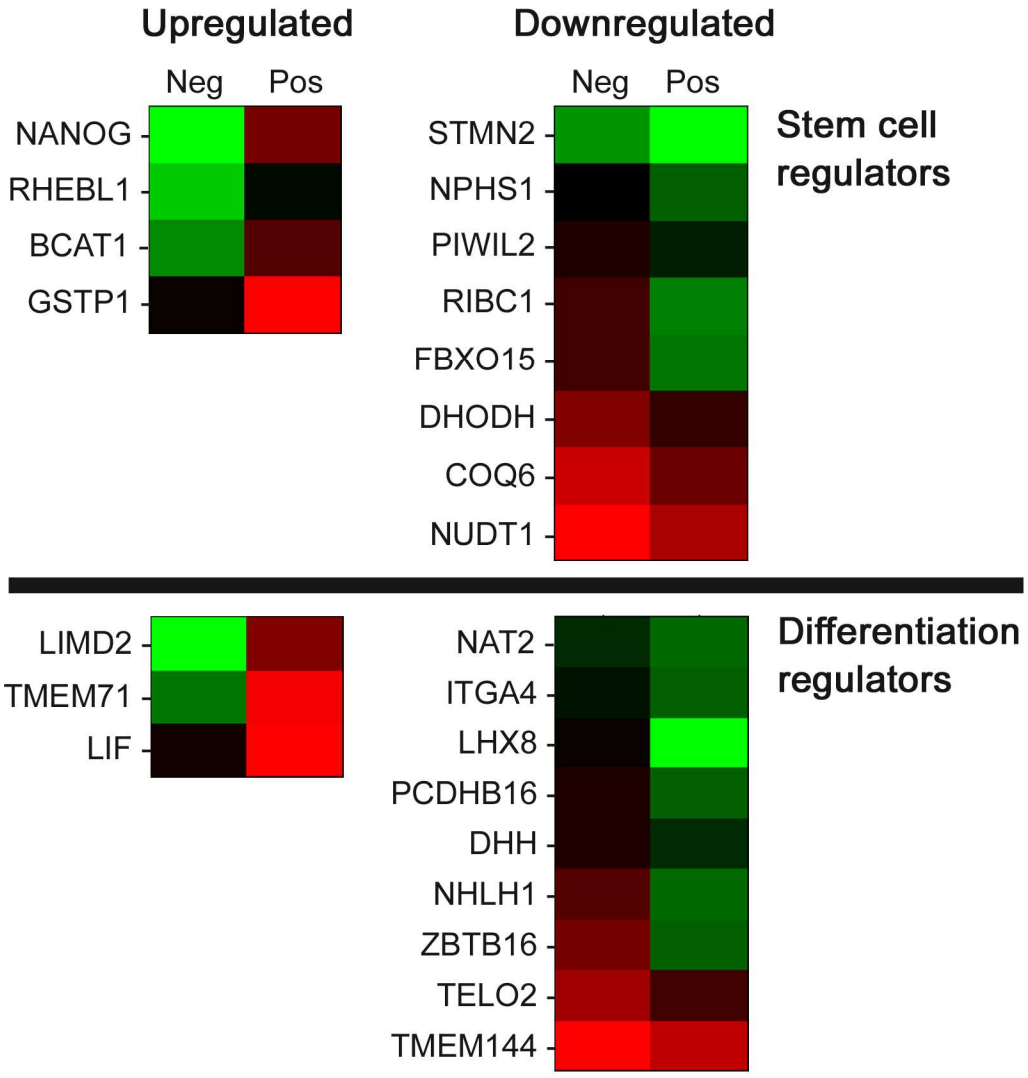
Supplementary Fig. 3. Increasing N-cadherin expression upon sequential passaging of LAPC9 xenografts in castrated mice, with double staining for N-cadherin and AR. Cell surface expression of N-cadherin (green) increased with passage number (P1 to P5), while the fraction of AR expressing cells (red) decreased. A majority of cells in P2 and 3 co-expressed both AR and N-cadherin. In later passages, distinct populations of N-cadherin^{+}/AR^{low} and AR^{+}/N-cadherin^{low} were visible.



Supplementary Fig. 4. N-cadherin staining of PC3 xenograft tumors with and without 2A9 treatment. Control tumors (left) showed clear cell surface staining, while antibody treated tumors (right) showed significantly less immunoreactivity.



Supplementary Fig. 5. Transient N-cadherin knockdown in PC3 cells caused decrease in expression of IL-6, IL-8, vimentin, TGF-β2, and VEGF, but did not restore expression of E-cadherin or AR. Gene expression was determined by semi-quantitative RT-PCR; LNCaP cell was included as positive control for E-cadherin and AR.



Supplementary Fig 6. Gene expression analysis of sorted N-cadherin positive and negative populations of LAPC9-CR xenografts. N-cadherin positive cells demonstrated upregulation of known stem cell markers (not shown) such as CD44, CD55, Sca1, and c-Met, as well as genes identified as regulators of embryonic stem cells including NANOG, RHEBL1, BCAT1, and GSTP1[22]. In contrast, N-cadherin positive cells showed downregulation of genes involved in differentiation of stem cells such as LHX8, ZBTB16, TMEM144, ITGA4, and NHLH1.

Figure 1

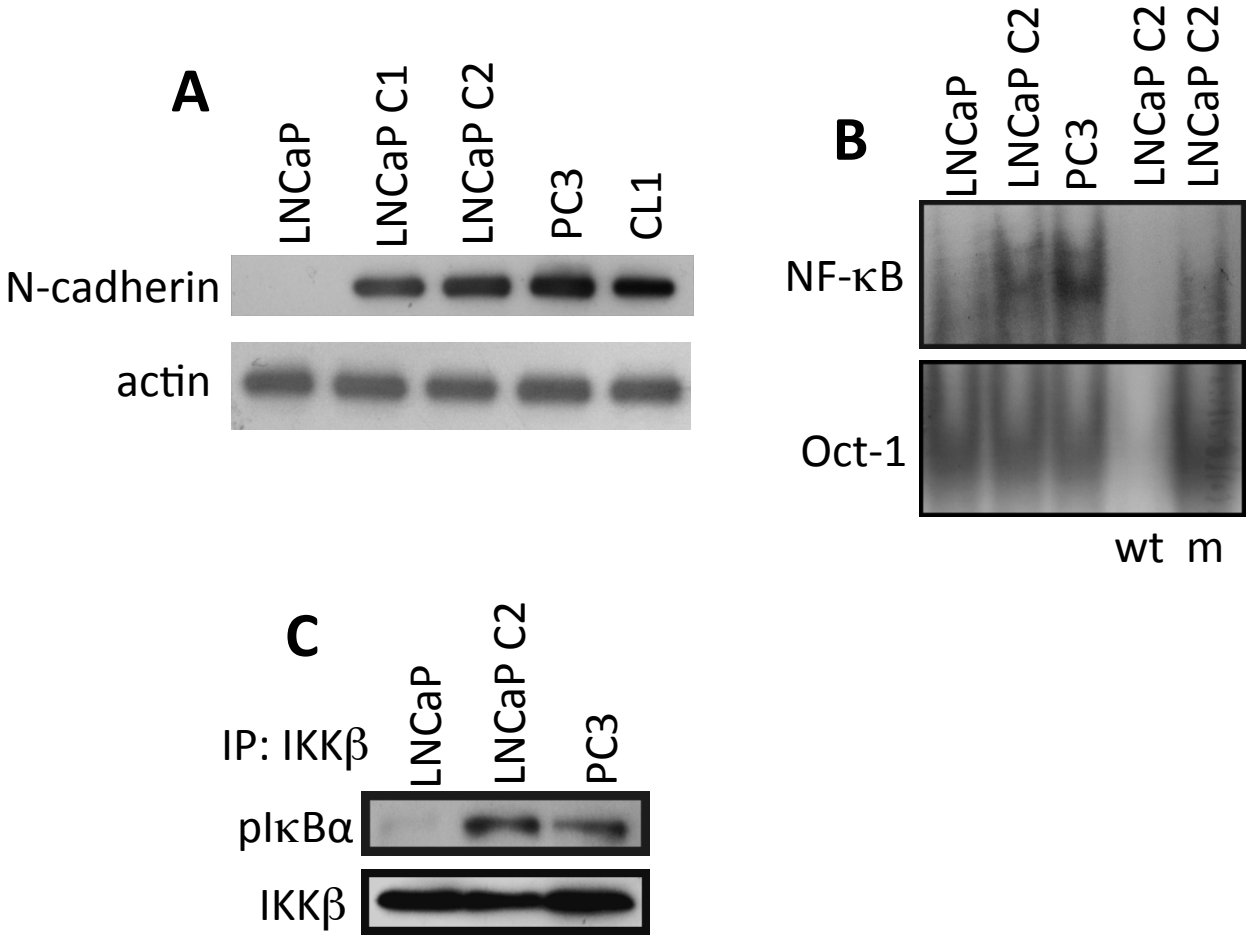


Figure 1. N-cadherin expression is associated with heightened constitutive NF-κB activity. A) Western blots for N-cadherin and actin (as a loading control). B) Electrophoretic mobility shifts assays (EMSAs) demonstrated heightened binding of NF-κB family members to a consensus double-stranded oligonucleotide NF-κB binding site. Bottom panel of EMSAs demonstrated lack of effect on an Oct-1 EMSA thereby illustrating specificity of N-cadherin effect. At right of EMSAs are cold competition experiments with cold wild-type (wt) and mutant (m) NF-κB probes. C) Constitutive IKKβ activation detected by *in vitro* kinase assays. IKKβ complexes were immunoprecipitated and phosphorylation of its substrate, IκB was determined by Westeron blot (top panel). Bottom panel is Western blot to demonstrate equal immunoprecipitation of IKKβ.

Figure 2

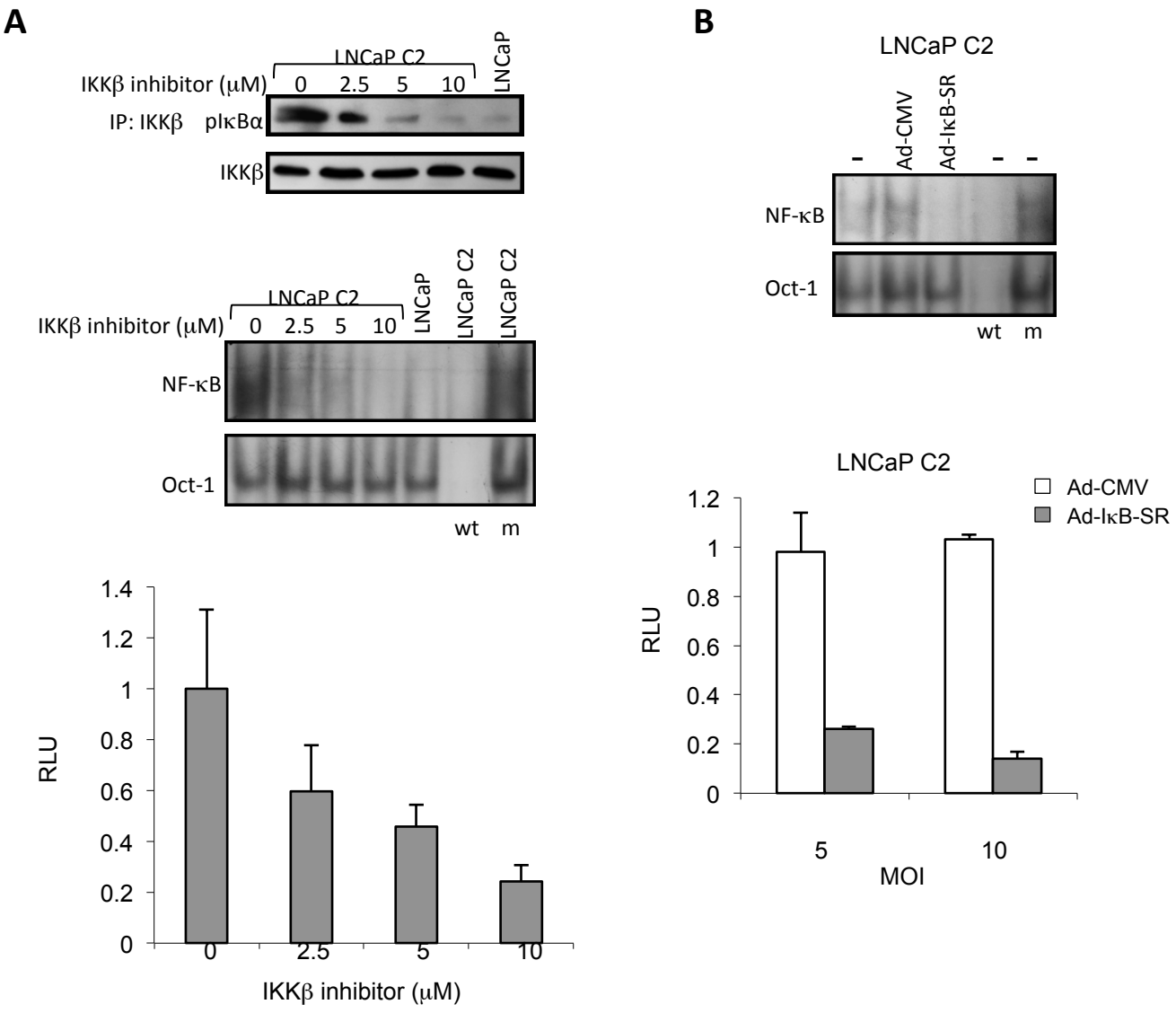


Figure 2. Inhibition of NF-κB by pharmacologic and molecular approaches. A) A pharmacologic inhibitor of IKKβ blocks NF-κB activation as measured by IKKβ *in vitro* kinase assays, EMSAs and NF-κB driven reporter gene activity (top, middle, and bottom panels, respectively). B) The dominant active inhibitor known as IκB-SR (in adenoviral vector) inhibits NF-κB activity measured by EMSAs and NF-κB driven reporter gene activity.

Figure 3

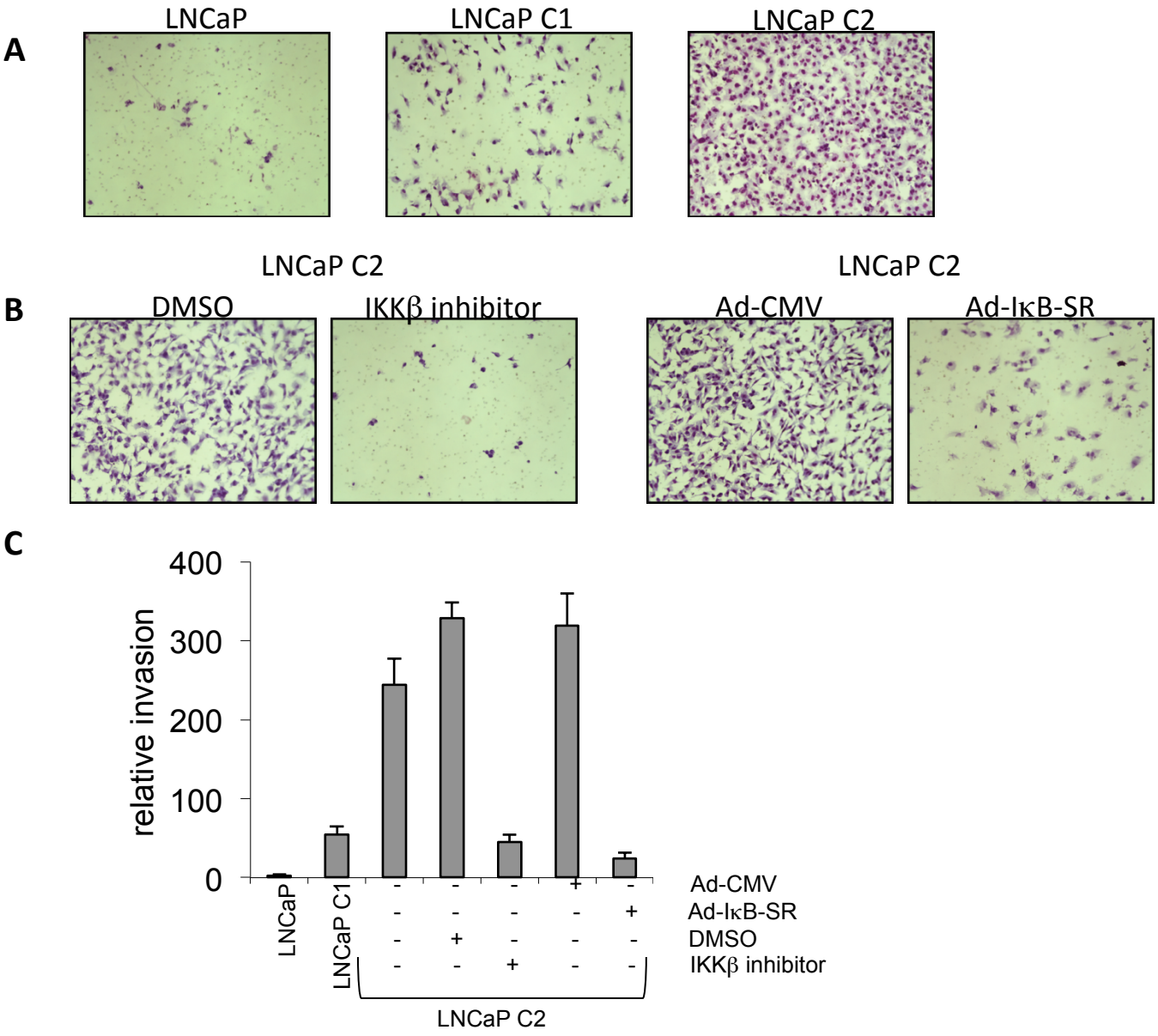


Figure 3. Inhibition of NF-κB results in reduced invasiveness of N-cadherin expressing cells. A) Photomicrographs illustrate heightened invasiveness by Matrigel invasion assay of LNCaP cells versus LNCaP-C1 and C2, which ectopically express N-cadherin. B) Inhibition of NF-κB by IKKβ inhibitor or the Ad-IκB-SR reduces invasiveness of LNCaP-C2 cells. C) Histogram of results from A and B. D)) Inhibition of NF-κB by IKKβ inhibitor or the Ad-IκB-SR induces E-cadherin expression.

Figure 4

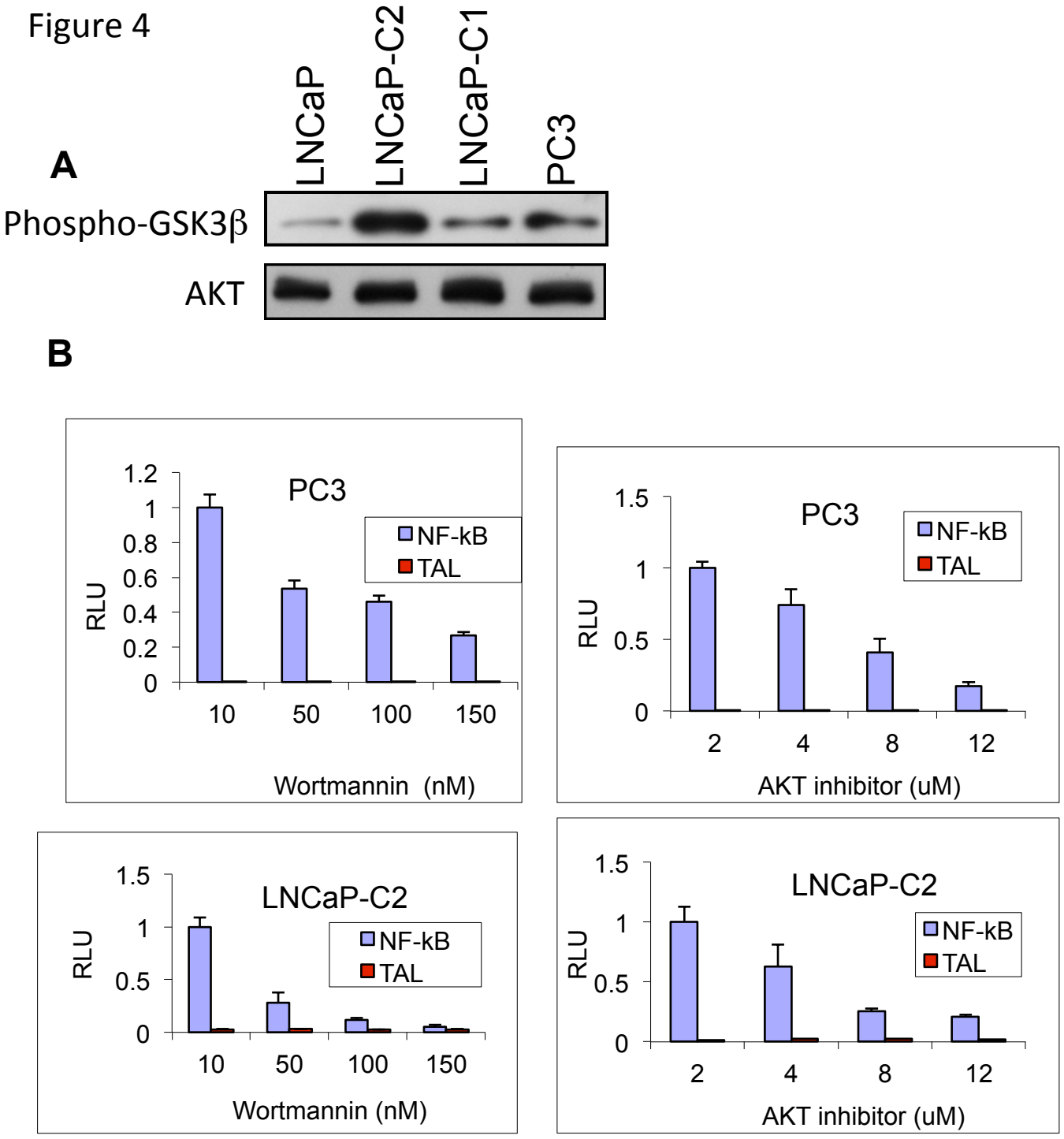


Figure 4. Heightened constitutive AKT activity in N-cadherin expressing cells drives NF-κB activation. A) Constitutive AKT activation is observed in N-cadherin expressing cells as measured by AKT *in vitro* kinase assays. Phosphorylation of the AKT substrate GSK3β by AKT immunoprecipitates is shown in the top panel and equal immunoprecipitation of AKT is shown in bottom panel. B) Inhibition of the PI3K/AKT pathway with a selective PI3K inhibitor (wortmannin) and AKT inhibitor reduces NF-κB reporter gene activity. Reporter gene activity was normalized to that of vehicle treated (DMSO) cells and transfection efficiency was normalized by co-transfection of a *Renilla* luciferase reporter (pRL-SV40). Results are means +/- standard deviation. Similar results were obtained for another PI3K inhibitor, LY294002 (not shown).

Figure 5

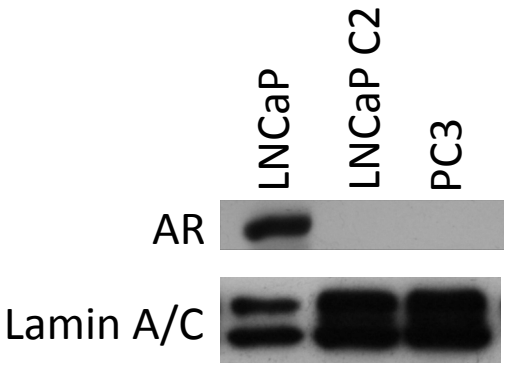


Figure 5. N-cadherin expression is associated with reduced AR expression. Nuclear extracts were subjected to immunoblotting with an anti-AR antibody and a Lamin A/C antibody (loading control for nuclear protein)

Figure 6

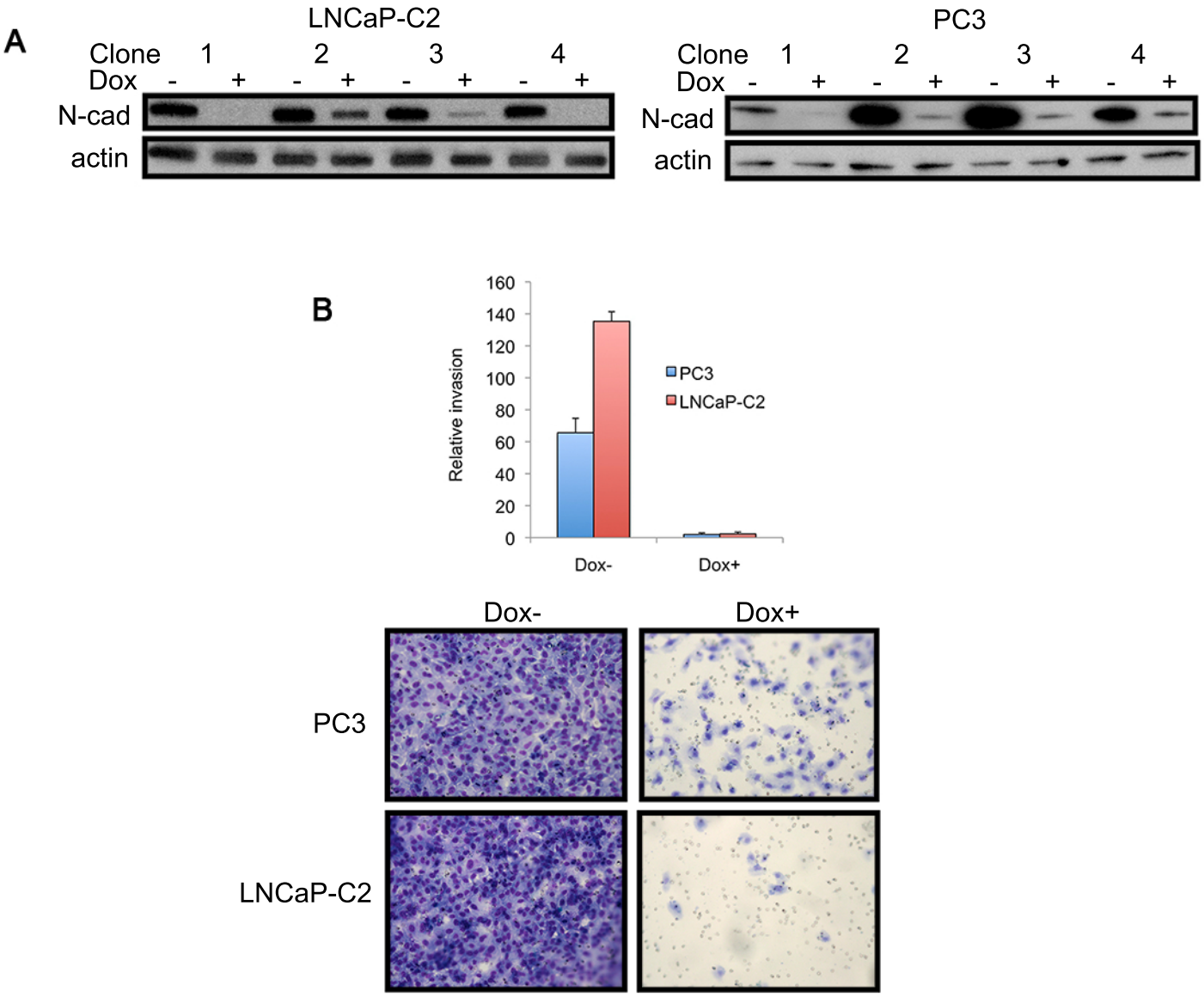


Figure 6. Inducible N-cadherin shRNA. A) LNCaP-C2 cells, which stably express N-cad, and PC3 cells, which endogenously express N-cad, were stably transduced with a lentivirus in which N-cad shRNA expression is tetracycine/oxycycline inducible. Shown are four clones for each cell line with N-cad expression depicted in the presence or absence of doxycycline (1 μg/ml). **B)** Invasion of LNCaP-C2 and PC3 cells is reduced upon induction of N-cad shRNA by doxycycline. The histogram at the top illustrates the marked difference in invasion in a Matrigel invasion assay; results are means of triplicates +/- s.d. Importantly, neither doxycycline nor N-cad shRNA affect the *in vitro* growth of cell lines during the time period of the assay, indicating that differences in invasion were *not* attributable to changes in rate of growth. Bottom panels are images of cells that have invaded into the Matrigel matrix (final magnification = 200X).

Figure 7

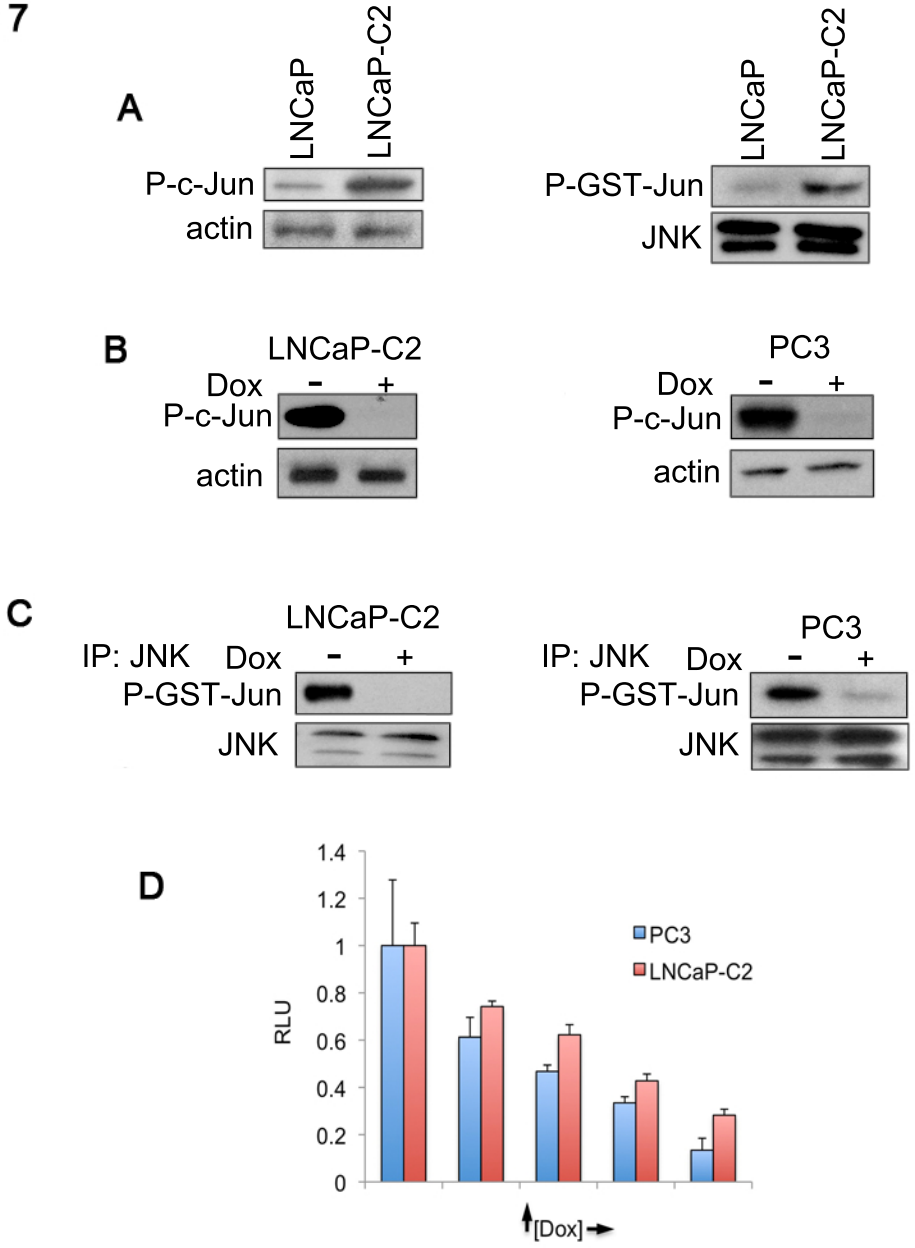


Figure 7. N-cadherin expression leads to activation of JNK/AP1. A) Increased phosphorylation of c-Jun (Ser 73) and JNK activity in LNCaP-C2 cells, which were engineered to stably express N-cad. Left panels are Western blots for the indicated proteins. Right panels represent a JNK *in vitro* kinase assay, in which JNK was immunoprecipitated; immunoprecipitates were subjected to a kinase assay, whereby phosphorylation of recombinant GST-c-Jun served as the read-out. **B)** N-cad silencing by inducing N-cad shRNA with doxycycline suppresses phosphorylation of c-Jun. **C)** Same as B) but read-out is JNK *in vitro* kinase assay. **D)** Increasing expression of N-cad shRNA by sequentially higher concentrations of doxycycline results in a dose-dependent reduction in AP1 reporter activity. Results are means of triplicates +/- s.d.

Figure 8

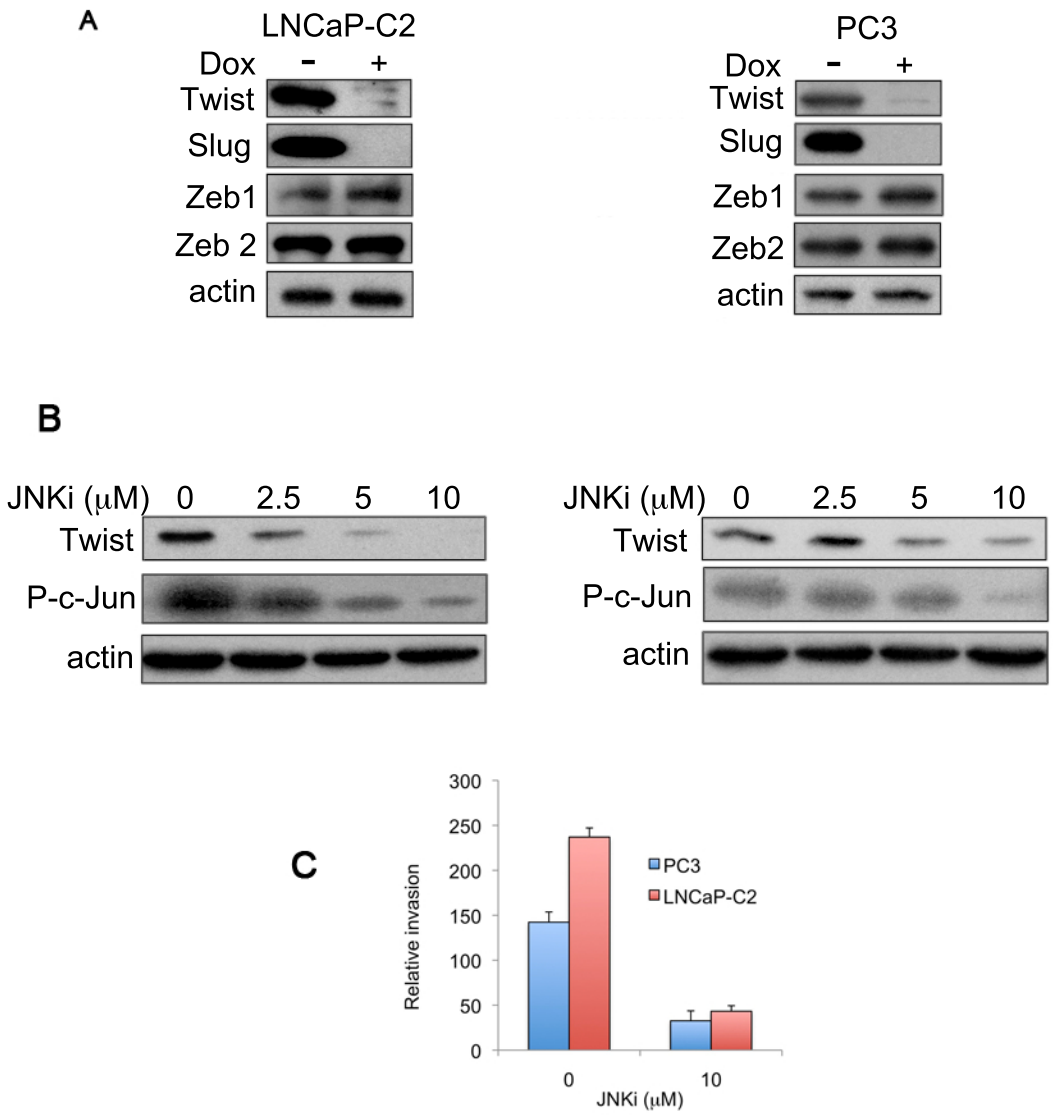


Figure 8. N-cadherin regulates Twist and Slug and invasion. A) Silencing N-cad expression through induction of N-cad shRNA with doxycycline reduced expression of Twist and Slug, but not Zeb1 or Zeb2. **B)** Twist expression is reduced by pharmacologic inhibition of JNK in a dose-dependent fashion by SP600125. **C)** SP600125 inhibits invasion in a Matrigel invasion assay. Results are means of triplicates +/- s.d.

Figure 9

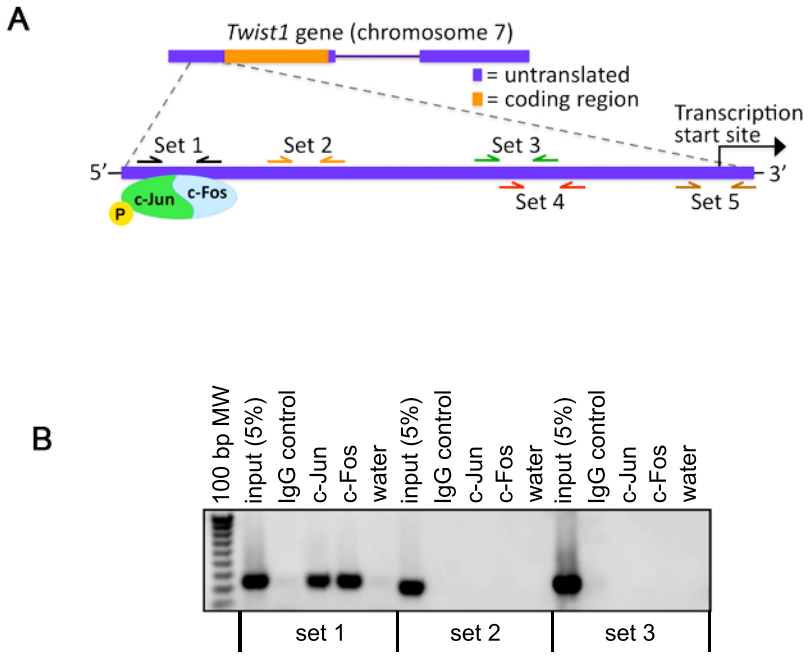


Figure 9. JNK/AP1-mediated regulation of *Twist* gene expression. A) Schematic figure of the *Twist* gene and promoter (modified from NCBI database). The *Twist* gene is located on chromosome 7p21. The ~2.5 kb upstream of the transcription start site was analyzed for putative AP1 binding sites with the aid of the M-Match search engine, and three PCR primers sets spanning predicted AP1 sites were generated for ChIP analysis. **B)** ChIP analysis of Twist promoter. DNA-protein extracts were immunoprecipitated with a c-Jun, c-Fos or IgG control antibody. Immunoprecipitates were subjected to PCR with five sets of primers covering various regions of the *Twist* promoter containing putative AP1 binding sites. PCR products are shown. PCR amplification of DNA not subjected to immunoprecipitation served as a positive control, and amplification of water was a negative control.

Figure 10

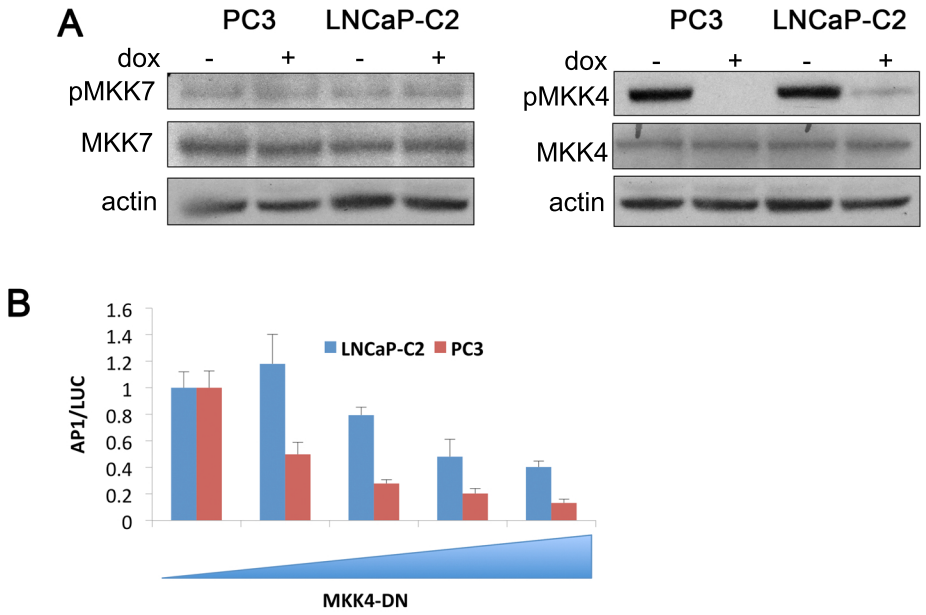


Figure 10. MKK4 and MKK7 activity in PC3 and LNCaP-C2 doxycycline inducible cell lines. A) Cells were treated with doxycycline (1 μg/mL) overnight and Western blots were performed for the indicated proteins. Actin serves as a loading control. B) A kinase deficient MKK4-dominant negative (MKK4-DN) was transietly co-transfected with an AP1-luciferase reporter construct LNCaP-C2 or PC3 cells, and 48 hours later, protein was harvested for firefly luciferase read-out. Co-transfection of a *Renilla* reporter construct served to normalize for transfection efficiency. Results are means of triplicates +/- s.d.

Figure 11

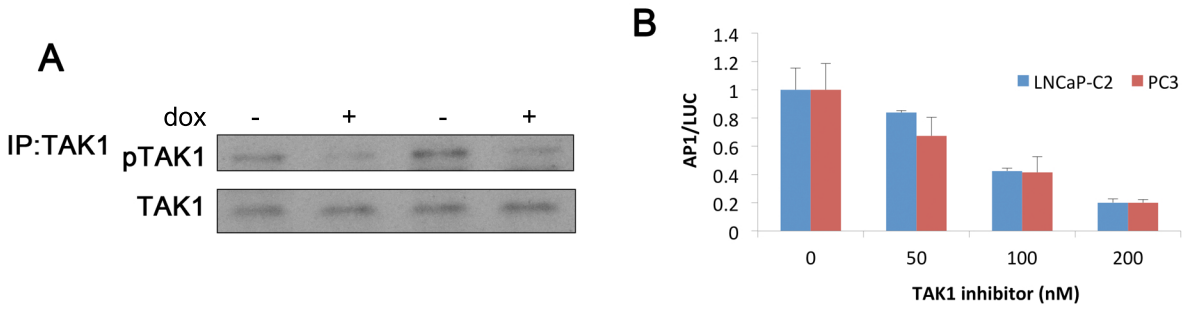


Figure 11. MKK4 and TAK1 regulate AP1 activity. A) Cells were treated with doxycycline (1 μg/mL) overnight and then TAK1 was immunoprecipitated followed by Western blots for the indicated proteins. Actin serves as a loading control. B) Cells were exposed to a commercially available TAK1 inhibitor [(5Z)-7-oxozeaenol;Tocris Bioscience] for 24 hours followed by co-transfection of an AP1 luciferase reporter construct and a *Renilla* reporter for transfection efficiency. 24 hours later, protein was extracted for luciferase assays. Results are means of triplicates +/- s.d.